THE GLUTATHIONE S-TRANSFERASES IN HUMAN LIVER CYTOSOL

BY

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A thesis submitted for the degree of
Doctor of Philosophy, University of Edinburgh.

1987

DECLARATION OF ORIGINALITY

I declare that the work presented herein and the composition of this thesis is my own.

Paul K. Stockman

ACKNOWLEDGEMENTS

I am most grateful to my supervisor, Dr J D Hayes for his advice and encouragement during my studies for this degree. I would also like to thank Professor L G Whitby, for critically reading the manuscripts which have been submitted for publication and for his advice concerning my studies in general.

Many colleagues have helped me during this work and it is a pleasure to thank them. Professor R P Ambler determined the amino acid compositions of GST; his assistance and advice have been very valuable. Mrs L I McLellan provided expert help with the peptide 'mapping' experiments. Dr G J Beckett supervised my experimental work using RIA and Miss A J Hussey assisted in preparing RIA reagents.

I am grateful to Professor L G Whitby and Professor R P Ambler for the use of their laboratory facilities in the University Department of Clinical Chemistry, Royal Infirmary, Edinburgh and in the University Department of Molecular Biology, King's Buildings, Edinburgh, respectively. I wish to thank Mrs E Ward for typing this thesis and also Mr I Lennox for the artwork.

This work was supported by a Medical Research Council Project Grant (G8126392SB) awarded to Dr J D Hayes. Travel, to the Biochemical Society Meeting and GST Workshop, Trinity College, Dublin, 23-29 September 1986, was generously supported by a Biochemical Society (Pharmacological Biochemistry Group) Bursary.

ABSTRACT

A purification scheme is described for the basic and neutral glutathione S-transferases which occur in human liver. Three forms with basic isoelectric points, B_1B_1 (pI 8.9), B_1B_2 (pI 8.75) and B_2B_2 (pI 8.4) and two forms with neutral isoelectric points, N_1 (pI 6.1) and N_2 (pI 4.6), were obtained. Not every liver examined expressed transferase N_1 or transferase N_2. An acidic enzyme from human lung, GST λ (pI 4.8), was included in the study for the purpose of comparison.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis demonstrated that each enzyme comprises two subunits of identical size. As a group the human glutathione S-transferases are composed of three distinct types of subunit with different molecular weights. The basic enzymes (B_1B_1, B_1B_2 and B_2B_2) were shown to have a subunit molecular weight of Mr 25 900, whereas, the neutral enzymes (N_1 and N_2) were found to comprise subunits of molecular weight Mr 26 500 and the acidic transferase from lung (λ) was shown to be composed of subunits of molecular weight Mr 24 800. Antisera were raised against each of these enzymes and they were shown to cross-react only with enzymes from the same charge/molecular weight group.

Contrary to previous work (Kamisaka *et al.*, (1975) Eur. J. Biochem. 60: 153-161) data are presented to demonstrate that two distinct basic subunits are expressed in human liver (B_1 and B_2). Reversible disso-

ciation and reassociation experiments demonstrated that transferases B_1B_1 and B_2B_2 could be formed from transferase B_1B_2. Tryptic-digest peptide 'maps' showed that the B_1 and B_2 subunits possess extensive sequence homology, however, seven peptides were recovered from transferase B_1B_1 that were not recovered from transferase B_2B_2. Conversely, four peptides were recovered from transferase B_2B_2 that were not recovered from transferase B_1B_1. However, all these peptides were recovered from transferase B_1B_2; this is consistent with the hypothesis that B_1B_2 is a hybrid enzyme.

A comparison of the substrate specificities of the enzymes demonstrated that transferases B_1B_1, B_1B_2 and B_2B_2 had a high peroxidase activity with cumene hydroperoxide, transferases N_1 and N_2 had a high activity with trans-4-phenyl-3-buten-2-one and transferase λ had a high activity with ethacrynic acid. The IC_{50} values obtained for the basic enzymes demonstrated that the B_1 subunit was much more potently inhibited by tributyltin acetate than the B_2 subunit.

The isoelectric point, molecular weight and high specific activity with trans-4-phenyl-3-buten-2-one of transferase N_1 suggest that it is identical to transferase μ, a form that has been previously characterised by Warholm et al., (1983) Biochemistry 22: 3610-3617. However, transferanse N_2 is a novel enzyme that has not been described previously. The properties of this new enzyme indicate that it also belongs to the neutral group of enzymes.

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SECTION 1: INTRODUCTION

1.1 HISTORICAL PERSPECTIVE: Mercapturic acids, glutathione and glutathione S-transferase

The first documented evidence of the catalytic activity associated with glutathione S-transferase (GST; EC 2.5.1.18) appeared in the late 19th century. Independently, the work of both Baumann & Preusse (1879), who investigated the metabolism of bromobenzene, and Jaffe (1879), who studied the metabolism of chlorobenzene, demonstrated that monohalogenobenzenes are excreted in urine as unstable N-acetylated S-substituted cysteine derivatives. These compounds have subsequently been called mercapturic acids and have been isolated from the urine of several species (reviewed by Jakoby & Habig, (1980).

The cysteine moiety of the mercapturic acids was considered to arise from a number of sources including dietary protein (Stekol, 1938), but Bray et al. (1959) conclusively established that the cysteine was derived from the tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH). The chemical properties of glutathione and its role in cellular metabolism are discussed in some detail in the next section.

In 1961 Booth et al. demonstrated the existence of an enzyme in rat liver cytosol that catalysed the conjugation of GSH with 1,2-dichloro-4-nitrobenzene (DCNB). The same year, work by Combes & Stakelum established the enzymic basis of the conjugation of GSH with bromosulphophthalein (BSP), a dye used in clinical tests for hepatic function.

It was apparent that the enzyme catalysing these reactions, later named glutathione S-aryltransferase (Grover & Sims, 1964) and more recently, glutathione S-transferase, was responsible for the first step in the synthesis of mercapturic acids. Subsequently, Jakoby and his colleagues established that glutathione S-transferase is represented by multiple isoenzymes each of which displays activity towards a different spectrum of xenobiotics (Habig et al., 1974b, 1976b).

The thioesters, formed by the reaction catalysed by the glutathione S-transferases, are converted to mercapturic acids in three separate stages by: (1) removal of the γ-glutamyl moiety through the action of a γ-glutamyltransferase; (2) removal of glycine by the action of a dipeptidase and; (3) N-acetyl-CoA linked acetylation of the cysteine conjugate to form a N-acetylcysteine thioether, which is the mercapturic acid (Barnes et al., 1959; Bray et al., 1959; Boyland & Chasseaud, 1969; Habig et al., 1974b). A schematic representation of this pathway is shown in Figure 1.1.

1.2 GLUTATHIONE

a. Chemical properties

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine; Fig. 1.2) is synthesised intracellularly from glutamate, cysteine and glycine and is probably the most abundant low molecular weight thiol. It is found in virtually all

Figure 1.1. Synthetic pathway of the mercapturic acids. The first step is catalysed by glutathione S-transferase. The glutatione thioether product may be excreted directly into the bile. Alternatively, if this conjugate reaches the kidney it is converted to the L-cysteine derivative by the sequential actions of γ-glutamyl transpeptidase and glycyl peptidase. Neutral amino acids are among the best acceptors for the γ-glutamyl moiety. The cysteinyl derivative can then be converted to a mercapturic acid by N-acetylation with acetyl CoA. Alternatively, if the cysteine conjugate is aromatic, it can be converted to the thiol derivative, pyruvate and NH_3 by the action of cysteine conjugate β-lyase. This enzyme occurs primarily in the liver and kidney.

Abbreviations: AA, amino acid; AcCoA, acetyl coenzyme A.

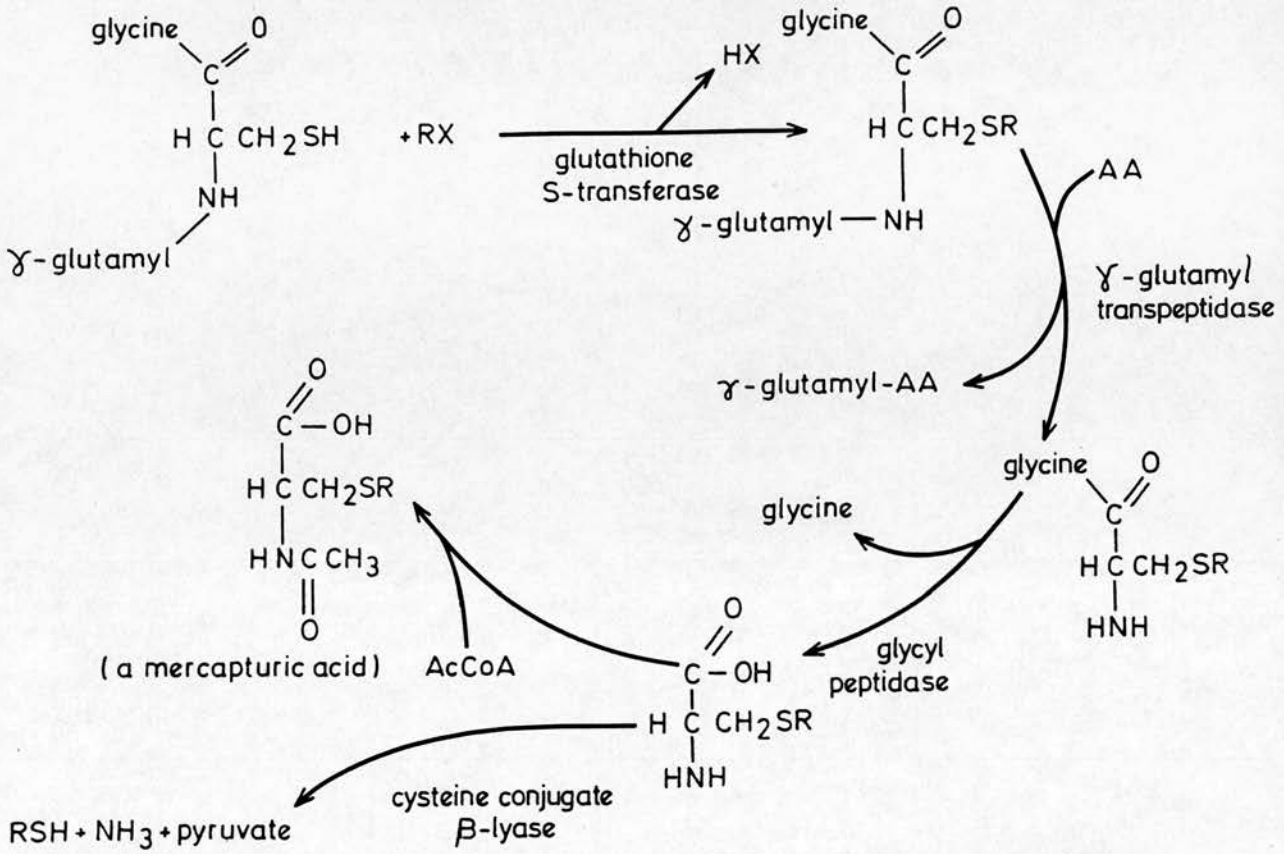

glycine
+RX
HX
glutathione
S-transferase
γ-glutamyl
AA
γ-glutamyl
transpeptidase
γ-glutamyl-AA
glycine
glycine
glycyl
peptidase
(a mercapturic acid)
AcCoA
cysteine conjugate
β-lyase
RSH + NH3 + pyruvate

Figure 1.1

aerobic cells in millimolar concentrations and at lower concentrations in extracellular fluid (Meister & Anderson, 1983).

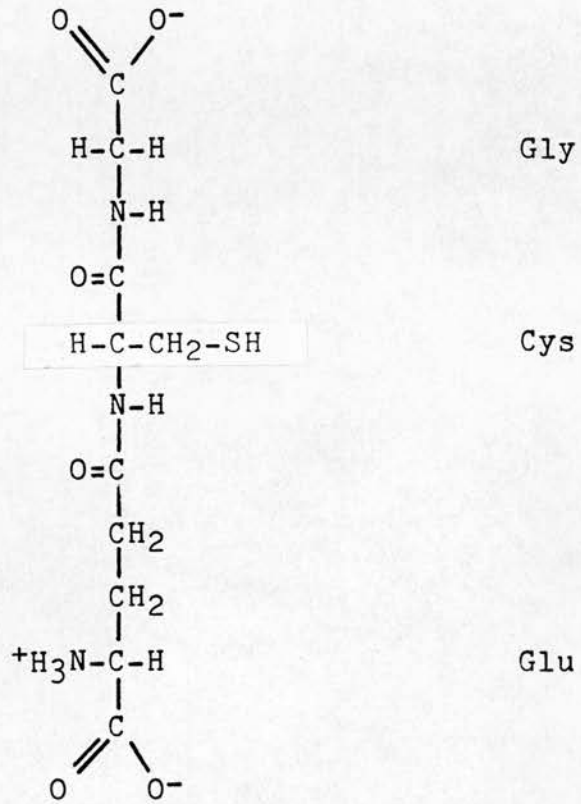


Figure 1.2. Structure of reduced glutathione.

It is widely recognised that glutathione, by its ability to form thioether conjugates with electrophilic compounds, assists in their elimination from the body (Jakoby, 1978a, 1978b; Chasseaud, 1979; Ketterer et al., 1983; Mannervik, 1985). The chemical properties of GSH are ideally suited in this role (Boyland & Chasseaud, 1969):-

The isoelectric point of glutathione is pH 2.8 and hence the molecule will have a net negative charge at physiological pH. It will, therefore, be hydrophilic and increase the solubility of hydrophobic moieties to which it may become conjugated.

2. The nucleophilic thiol group present in the cysteinyl residue is required for formation of glutathione-conjugates. This group has pKa value of about 9.3 (Habig, 1983) and will be protonated and less likely to react with potential substrates at physiological pH. Glutathione S-transferases are thought to activate the cysteinyl thiol moiety when they bind glutathione at the active site. The pKa of the thiol group is lowered, making it more nucleophilic and therefore more reactive to the second substrate which is located in an adjacent hydrophobic binding site (Keen et al., 1976; Jakoby, 1978a, 1978b).
3. The γ-glutamyl peptide bond between the N-terminal glutamate and the cysteine residue of glutathione cannot be cleaved by alpha-carboxypeptidase but can be by γ-glutamyl transpeptidase. The latter enzyme catalyses the cleavage of the glutamate residue from the glutathione-conjugate; the first step in mercapturic acid synthesis.
4. The low molecular weight of GSH (Mr 307.3) ensures that the conjugate will be efficiently cleared via the biliary system (Hirom et al., 1972).

The cellular functions of GSH are diverse. It is a co-enzyme for several reactions including those catalysed by glyoxylase I and formaldehyde dehydrogenase and is a substrate for a number of enzymes including glutathione S-

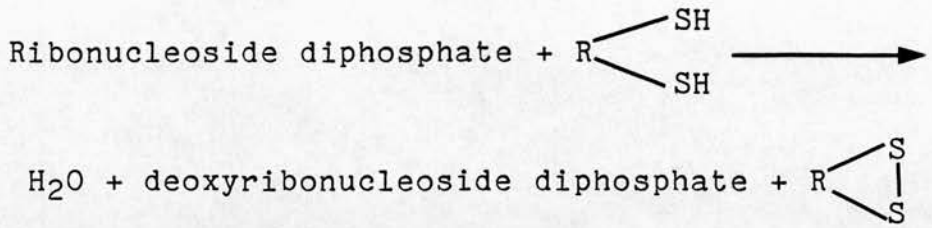
transferase, glutathione peroxidase and dehydroascorbate reductase (for a review see Meister & Anderson, 1983). Glutathione metabolism is closely linked with the transport of amino acids into cells, it participates in the reduction of disulphides and other molecules, conjugates with compounds of exogenous and endogenous origin and protects cells against the destructive effects of reactive oxygen intermediates and free radicals.

1.2b. Glutathione as an intracellular reductant

Glutathione exists in reduced (GSH) and oxidised forms (GSSG) and is the major thiol redox system of the cell. Most glutathione is present *in vivo* as GSH but up to one-third of the total cellular glutathione may be present as "mixed" disulphides with other compounds that contain -SH groups such as cysteine, coenzyme A, and the -SH of the cysteine residues of protein molecules. Mixed disulphides are formed in a reaction catalyzed by thiol transferase (Eriksson *et al.*, 1974):

protein-SH + GSSG $\rightleftharpoons$ protein-SSG +GSH.

Glutathione can provide the reducing capacity for cellular reactions (Meister, 1983), for example, the reduction of ribonucleosides. Deoxyribonucleotides, the precursors of DNA, are formed *in vivo* by the reduction of ribonucleoside diphosphate reductase. This enzyme catalyses replacement of the 2'-OH group on the ribose sugar by a hydrogen atom:



Where $R(SH)_2$ is the dithiol compound thioredoxin. This small protein has two cysteine -SH groups in close proximity. Oxidised thioredoxin (RS_2) is returned to the reduced state at the expense of NADPH. In certain tissues such as calf thymus and E.coli, a related protein glutaredoxin, fulfils a similar role to thioredoxin. However, in these tissues the oxidation of glutaredoxin is achieved at the expense of GSH, which is oxidised to GSSG (Halliwell & Gutteridge, 1985a; Fig. 1.3).

The cellular redox state is determined by the distribution of glutathione between its oxidised and reduced forms. In normal cells glutathione is maintaned predominantly in the reduced state (the GSH:GSSG ratio is 250:1 in liver) and this relationship between the two forms has an important effect on the oxidation/reduction state of protein thiols. Indeed, high concentrations of GSSG can inhibit protein synthesis and a number of enzymes such as adenylate cyclase and phosphorlyase phosphatase by forming "mixed" disulphides with them (Kosower & Kosower, 1976):

$$\text{enzyme-SH} + \text{GSSG} \rightleftharpoons \text{enzyme-S-S-G} + \text{GSH}$$

These observations have led Gilbert (1982) to suggest that glutathione could act as a "third messenger",

Figure 1.3. Reduction of ribonucleoside diphosphates by GSH.

The enzyme, ribonucleoside diphosphate reductase catalyses the conversion of ribonucleoside-5'- diphosphate to deoxyribonucleoside -5'-diphosphate. In most tissues the reaction proceeds by oxidation of thioredoxin. The oxidised thioredoxin is then oxidized by a reductase enzyme at the expense of NADPH. By contrast, in E.coli and in calf thymus an additional protein, glutaredoxin, can also donate electrons to ribonucleoside diphosphate reductase. Glutaredoxin, however, is oxidized at the expense of GSH. The GSSG so produced is reduced by glutathione reductase.

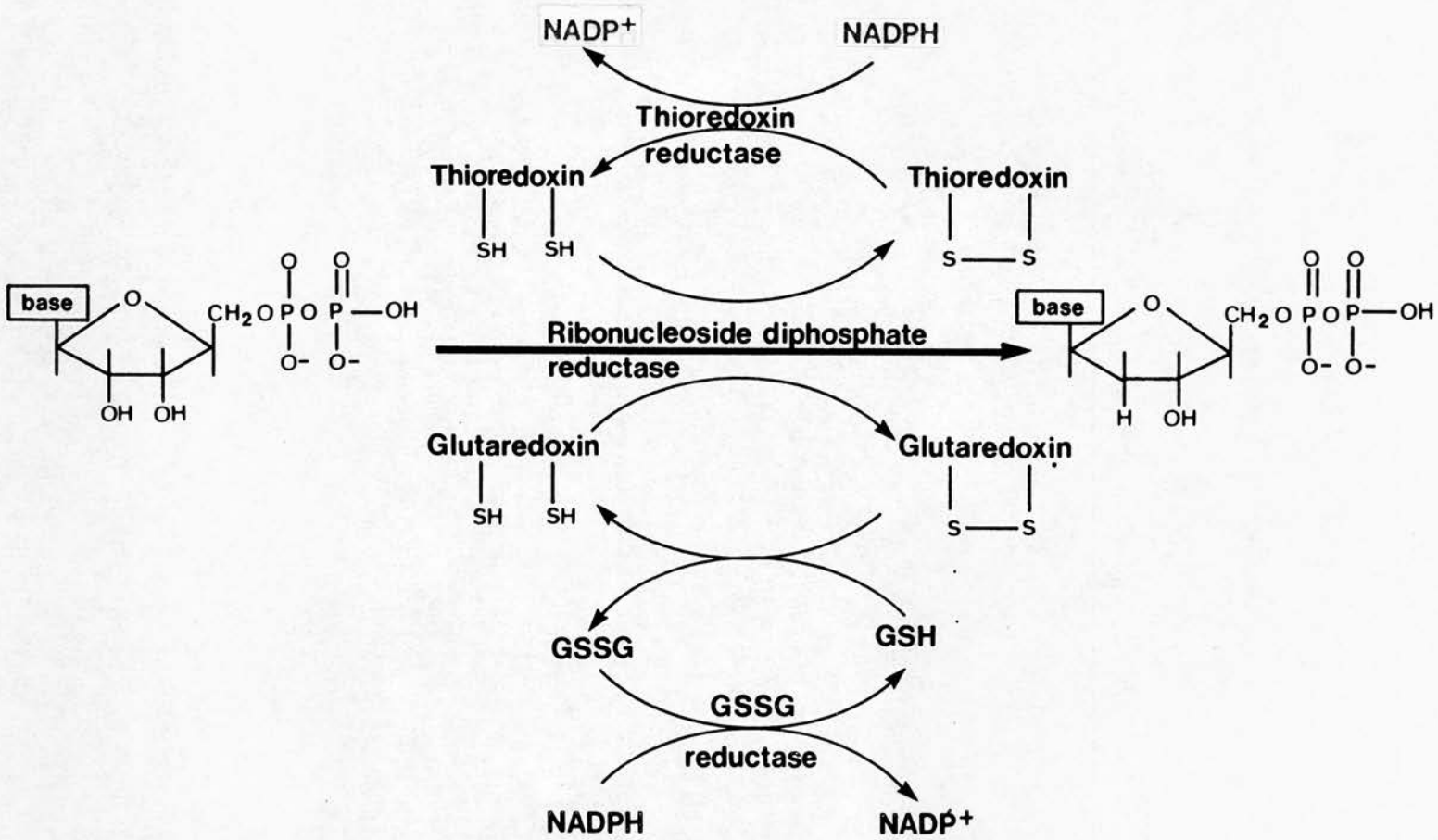

NADP+
NADPH
Thioredoxin reductase
Thioredoxin
SH SH
Thioredoxin
S—S
base
CH2O P O P—OH
O- O-
OH OH
Ribonucleoside diphosphate reductase
base
CH2O P O P—OH
O- O-
H OH
Glutaredoxin
SH SH
Glutaredoxin
S—S
GSSG
GSH
GSSG reductase
NADPH
NADP+

Figure 1.3

mediating certain activities of cells.

Glutathione reductase enzymes maintain the high GSH:GSSG status in normal cells at the expense of NADPH:

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

These reductases can also catalyse the reduction of some mixed disulphides, for example, that between GSH and co-enzyme A.

1.2c. Glutathione: its protective role against oxygen toxicity and free radicals

Oxygen, although essential to the existence of aerobic organisms is also potentially harmful to them through their capacity to generate O_2^- radicals which can be transformed into the very active, membrane peroxidising hydroxyl radical ($OH^\cdot$). The most important sources of O_2^- *in vivo* are the electron transport chains of mitochondria and the endoplasmic reticulum. In most cells, O_2^- is rapidly dismutated to hydrogen peroxide by superoxide dismutase:

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide is damaging in living systems since it can give rise to the formation of $OH^\cdot$ radicals which react with cellular components to produce organic radicals.

Catalase and glutathione peroxidases are of major importance in the removal of hydrogen peroxide and organic peroxides from cells, although the relative importance of these two enzyme systems varies from tissue to tissue.

Two types of glutathione peroxidase have been identified in animal tissues (Prohaska & Ganther, 1977): The selenium-dependent glutathione peroxidase (Type I) and the selenium-independent peroxidase (Type II).

Selenium-dependent glutathione peroxidase contains an atom of selenium at its active site and occurs in the cytosol and mitochondria of animal tissues. It catalyses the reaction with hydrogen peroxide:

$$2\ GSH + H_2O_2 \longrightarrow 2\ H_2O + GSSG$$

but will also act on a range of organic peroxides such as cumene hydroperoxide, thymine peroxide and lipid peroxides. In each case, the peroxides (ROOH) are reduced to alcohols (ROH).

Selenium-independent glutathione peroxidase acts on organic peroxides but not on hydrogen peroxide. This activity is associated with certain types of glutathione S-transferase. The relative contribution of selenium-dependent and independent peroxidase activities varies in different organs of the rat (Halliwell & Gutteridge, 1985b). Glutathione can also act directly to inactivate reactive organic radicals generated by the action of $OH^{\cdot}$. The equation for the reaction where $R^{\cdot}$ denotes the organic radical:

$$R^{\cdot} + GSH \longrightarrow RH + GS^{\cdot}$$

$$2GS^{\cdot} \longrightarrow GSSG$$

1.2d. Metabolism of Glutathione: the γ-glutamyl cycle and conjugation

The series of reactions which catalyse the synthesis and degradation of glutathione are closely linked with the transport of glutathione out of cells and of γ-glutamyl amino acids into cells. This process represents the " γ-glutamyl cycle" and has been elucidated largely by Meister and his colleagues (Meister & Tate, 1976) and is summarized in Figure 1.4.

Glutathione is synthesized intracellularly from glutamate, cysteine and glycine, catalysed by the sequential actions of γ-glutamylcysteine synthetase and glutathione synthetase. The major site of GSH synthesis is the liver. The tripeptide is then transported out of the cell. Glutathione liberated from the liver is a substrate for the membrane bound enzyme γ-glutamyltranspeptidase found in high concentration in the kidney. This enzyme catalyses the transfer of the γ-glutamyl moiety of GSH (and also GSSG) to amino acid acceptors, to form γ-glutamyl amino acids and cysteinylglycine (CysH-Gly). Meister proposed that these γ-glutamyl amino acids are formed at the cell membrane and transported into the cell. Thus the γ-glutamyl moiety serves as a carrier for the transport of amino acids into the cell.

An intracellular enzyme, γ-glutamyl cyclotransferase, catalyses the conversion of γ-glutamyl amino acids to 5-oxoproline, liberating the free amino acids. 5-Oxo-

Figure 1.4. Outline of glutathione metabolism
(a) The γ-glutamyl cycle.

The cellular turnover of GSH involves intracellular synthesis from glutamate, cysteine and glycine catalysed by the sequential actions of γ-glutamyl cysteine synthetase (1) and GSH synthetase (2). The tripeptide is transported from the cell (3) and converted by membrane-bound γ-glutamyl transpeptidase (4) to cysteinylglycine (CysH-Gly) and γ-glutamyl amino acids. Cysteinylglycine is cleaved to cysteine (CysH) and glycine (Gly), catalysed by membrane bound dipeptidase or may occur intracellularly after transport of the dipeptide (5). Transported γ-glutamyl amino acids (6) are converted by γ-glutamyl cyclotransferase (7) to amino acids and 5-oxoproline. 5-oxoproline is decyclized by 5-oxoprolinase (8) to glutamate.

(b) Conjugation reactions.

Glutathione is conjugated with a variety of compounds of exogenous and endogenous origin, (X) in reactions catalysed by glutathione S-transferases (9). The conjugates are transported (10) and follow similar pathways to glutathione (4), (6) and (11). S-Substituted derivatives of cysteine are acetylated (12) to form mercapturic acids, which are transported out of the cell (13).

(c) Intracellular reduction reactions.

Intracellular GSH is converted to GSSG in thiol disulphide exchange reactions (14) with protein disulphide bonds and low molecular weight disulphides in reactions catalysed by glutathione peroxidases (15) and by reaction with free radicals (16). Glutathione reductase (17) catalyses the formation of GSH from GSSG. Adapted from Meister, (1983).

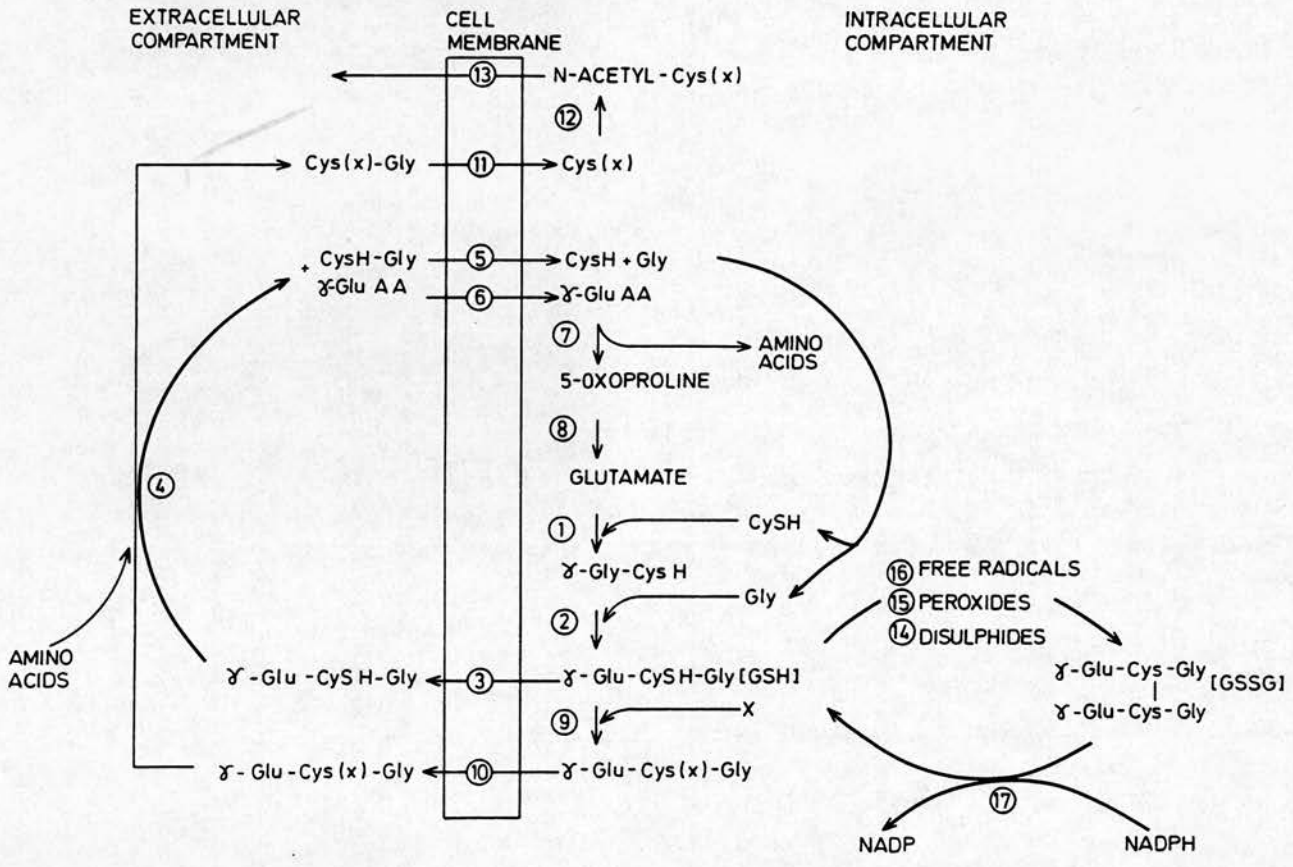

EXTRACELLULAR COMPARTMENT
CELL MEMBRANE
INTRACELLULAR COMPARTMENT
N-ACETYL - Cys (x)
Cys (x)-Gly
Cys (x)
CysH-Gly
γ-Glu AA
CysH + Gly
γ-Glu AA
AMINO ACIDS
5-OXOPROLINE
GLUTAMATE
CySH
γ-Gly-Cys H
Gly
FREE RADICALS
PEROXIDES
DISULPHIDES
AMINO ACIDS
γ-Glu -CyS H-Gly
γ-Glu-CyS H-Gly [GSH]
X
γ-Glu-Cys-Gly
γ-Glu-Cys-Gly
[GSSG]
γ-Glu-Cys (x)-Gly
γ-Glu-Cys (x)-Gly
NADP
NADPH

Figure 1.4

proline is converted by 5-oxoprolinase to glutamate in a reaction requiring the hydrolysis of ATP.

Cysteinylglycine, formed by the action of γ-glutamyl-transpeptidase is cleared by the action of a membrane bound dipeptidase and the free amino acids are then transported into the cell.

A wide range of electrophilic compounds are conjugated with glutathione, catalysed by the glutathione S-transferases. The conjugates, like glutathione itself, can be transported across cell membranes and follow pathways similar to those involved in GSH turnover. S-substituted cysteine derivatives are acetylated to form mercapturic acids which are transported out of cells and ultimately excreted in urine or faeces.

1.3 GLUTATHIONE S-TRANSFERASE (GST)

a. Purification and nomenclature of GST

Following the initial work by Boyland and his colleagues (Booth et al., (1961) into the specificity of GST, four types of activity were described; glutathione S-alkyl and S-aryltransferase, glutathione S-epoxide transferase and glutathione S-alkene transferase (Boyland & Chasseaud, 1969). While none of the enzymes responsible for these activities had been purified, this substrate-based nomenclature was widely adopted. However, this classification of GST based on the reactive group or carbon skeleton of the substrates was found to be ambiguous once purified enzymes were available.

The laboratories of Jakoby & Mannervik separately demonstrated that homogenous preparations of GST had broad overlapping substrate specificities (Fjellstedt et al., 1973; Pabst et al., 1973; Habig et al., 1974b, 1976b; Askelof et al., 1975). However, the purification scheme devised by Jakoby and his colleagues (Habig et al., 1976b) proved to be the most influential since at that time it was the most comprehensive and provided the basis for a new nomenclature.

Habig et al. (1974b, 1976b) described a purification scheme that separated several forms of glutathione S-transferase from rat liver. The scheme devised involved passing a cytosolic extract through a DEAE-cellulose anion exchanger that was equilibrated with 10 mM Tris/HCl buffer pH 8.1. The material that was not retained by the exchanger was applied to CM-cellulose which was equilibrated with 10 mM sodium phosphate pH 6.7. Four enzyme forms were retained by CM-cellulose and were eluted with a linear salt gradient; these enzymes were designated AA, A, B and/or C based on their reverse order of elution. Two other forms D and E were reported not to bind to either ion-exchanger and were recovered in the flow-through fractions from CM-cellulose. Unfortunately, this purification procedure does not resolve all the GST forms which occur in rat liver nor those in other tissues. Thus the GST nomenclature based on this method is incomplete.

The subsequent development of affinity chromatography matrices such as S-hexylglutathione-Sepharose (Guthenberg et al., 1979) and glutathione-Sepharose (Simons & Vander Jagt, 1977) has greatly facilitated the purification of these enzymes. In addition, the recent application of chromatofocusing has met with great success in this field and has allowed many new GST forms to be isolated and characterized (Mannervik & Jensson, 1982; Koskelo & Icen, 1984).

Cytosolic and microsomal forms of GST have been described. The cytosolic enzymes are all dimeric. At least 12 GST forms have been described in rat liver (Gillham, 1973; Hayes & Chalmers, 1983; Hayes, 1984; Meyer et al., 1984). These enzymes comprise two subunits and are coded for by a limited number of genes. Hetero- and homo-dimers exist, and the large number of forms that have been isolated arise as the result of subunit hybridization (Hayes et al., 1981; Beale et al., 1982, 1983; Mannervik & Jensson, 1982; Hayes, 1983, 1984).

It is recognised that the GST forms in the rat and other species are best defined by their subunit composition. Currently, the most widely used nomenclature is that which defines the subunits according to their mobility when subjected to discontinuous sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (SDS/PAGE). Bass et al. (1977) defined the Ya, Yb and Yc glutathione S-transferase subunits by their mobility during SDS/PAGE.

As the molecular basis for the multiplicity of this group of enzymes has become clear, other workers have added further subunits to the nomenclature (i.e. Yf, Yk and Yn (Hayes & Mantle 1986b). This will be dealt with in greater detail in section 1.6 . The method of SDS/PAGE most widely used is that described by Laemmli (1970), but it should be noted that the degree of polymer cross-linking (produced by N,N'-methylenebisacrylamide) can greatly influence the relative mobility of different subunits (Hayes & Mantle, 1986c).

1.3b. The types of reaction catalysed by GST

As a group of enzymes, the glutathione S-transferases demonstrate several types of catalytic activity and accept a large spectrum of structurally diverse compounds as substrates. This information is summarised below. The substrate specificity of individual GST isoenzymes is discussed in section 1.6.

The reactions catalysed by GST generally involve nucleophilic attack by GSH on various organic electrophiles. The electrophilic centres can be carbon, nitrogen, sulphur or oxygen and these form the basis of the classification described below.

1. Reaction at electrophilic carbon:

   (a) Conjugation of glutathione with a substrate by the formation of a thioether linkage. This reaction can proceed by: i) substitution of a halogen leaving group. Substrates which fall into this

category include aryl halides such as 1-chloro-2, 4-dinitrobenzene (Fig. 1.5a) and bromosulphophthalein or alkyl halides (Fig. 1.5b) such as iodomethane (Keen et al., 1976); ii) addition reactions of GSH with epoxide groups (Fig. 1.5c) such as that of 1,2-epoxy-3-(p-nitrophenoxy)-propane and benzo[a]pyrene-4,5-oxide (Nemoto et al., 1975); iii) addition reactions of GSH with activated alkenes (α ,β -unsaturated carbonyl compounds) (Fig. 1.5d) such as ethacrynic acid and 4-hydroxyalk-2-enals (Alin et al., 1985a).

(b) Thiolysis reactions (Keen & Jakoby, 1978), for example:

p-nitrophenyl acetate + GSH ⟶ p-nitrophenol + S-acetylglutathione

(c) Steroid isomerisation (Benson et al., 1977). In these reactions GSH is not consumed and acts as a co-enzyme. GST catalyses the isomerisation in the formation of α, β -unsaturated Δ^4-3-keto-steroids from Δ^5-3-ketosteroids (Fig. 1.5e). A physiologically important steroid substrate is Δ^5-androstene-3,17-dione, which undergoes the reaction:

Δ^5-androstene-3,17-dione ⟶ Δ^4-androstene-3,17-dione

(d) Prostaglandin isomerisation. GST activity has been associated with the formation of prosta-

glandin D_2 from prostaglandin endoperoxides (Hayakawa et al., 1977) (Fig. 1.5f).

2. Reaction with electrophilic nitrogen.

GST can catalyse the reduction of organic nitrates. This nitroreductase activity was first reported by Habig et al. (1975) and involves substrates such as nitroglycerin and erythrityl tetranitrate. The reaction is thought to proceed with the formation of a sulphenyl nitrite intermediate ($GSNO_2$) which is attacked non-enzymatically by GSH to release nitrite (NO_2^-) and disulphide:

$$RONO_2 + GSH \xrightarrow{\text{enzymatic}} ROH + (GSNO_2)$$

$$(GSNO_2) + GSH \xrightarrow{\text{non-enzymatic}} GSSG + HNO_2$$

overall reaction:

$$RONO_2 + 2\ GSH \longrightarrow ROH + GSSG + HNO_2$$

3. Reaction with electrophilic sulphur (Keen et al., 1976)

(a) GST catalyse the nucleophilic attack by GSH on the sulphur atom of organic thiocyanates resulting in the formation of HCN and a mixed disulphide:

$$RSCN + GSH \longrightarrow GSSR + HCN$$

Both aryl and alkyl thiocyanates are substrates.

(b) Thiol-disulphide interchange can be catalysed by GST.

$$RSSR' + GSH \longrightarrow GSSR + R'SH$$

Hydrophobic disulphides such as L-cystine dimethyl ester are good substrates for this GST activity (Keen & Jakoby, 1978).

4. Reaction with electrophilic oxygen.

Organic peroxide reduction is catalysed by GST (Burk et al., 1978):

$$ROOH + 2\ GSH \longrightarrow ROH + GSSG + H_2O$$

The reaction is thought to occur in two stages analogous to those of the nitroreductase reaction. The first stage involves a thioperoxide intermediate (GSOR) which is attached non-enzymatically to form disulphide:

$$ROOH + GSH \xrightarrow{\text{enzymatic}} ROH + (GSOH)$$

$$(GSOH) + GSH \xrightarrow{\text{non-enzymatic}} GSSG + H_2O$$

This activity of GST is referred to as selenium-independent glutathione peroxidase activity or Type II glutathione peroxidase activity, to distinguish it from the activity catalysed by the seleno-enzyme, glutathione peroxidase (Type I) (Mills, 1957).

The structure of some commonly used GST substrates is given in Figure 1.6 .

Figure 1.5. Types of reaction catalysed by GST.

a. Conjugation of glutathione with an aryl halide (1-chloro-2,4-dinitrobenzene).

b. Conjugation of glutathione with an alkyl halide.

c. Conjugation of glutathione with an epoxide group.

d. Conjugation of glutathione to activated alkenes.

e. Ketosteroid isomerase activity.

f. Prostaglandin isomerase activity.

Reaction mechanisms adapted from review by Douglas (1987).

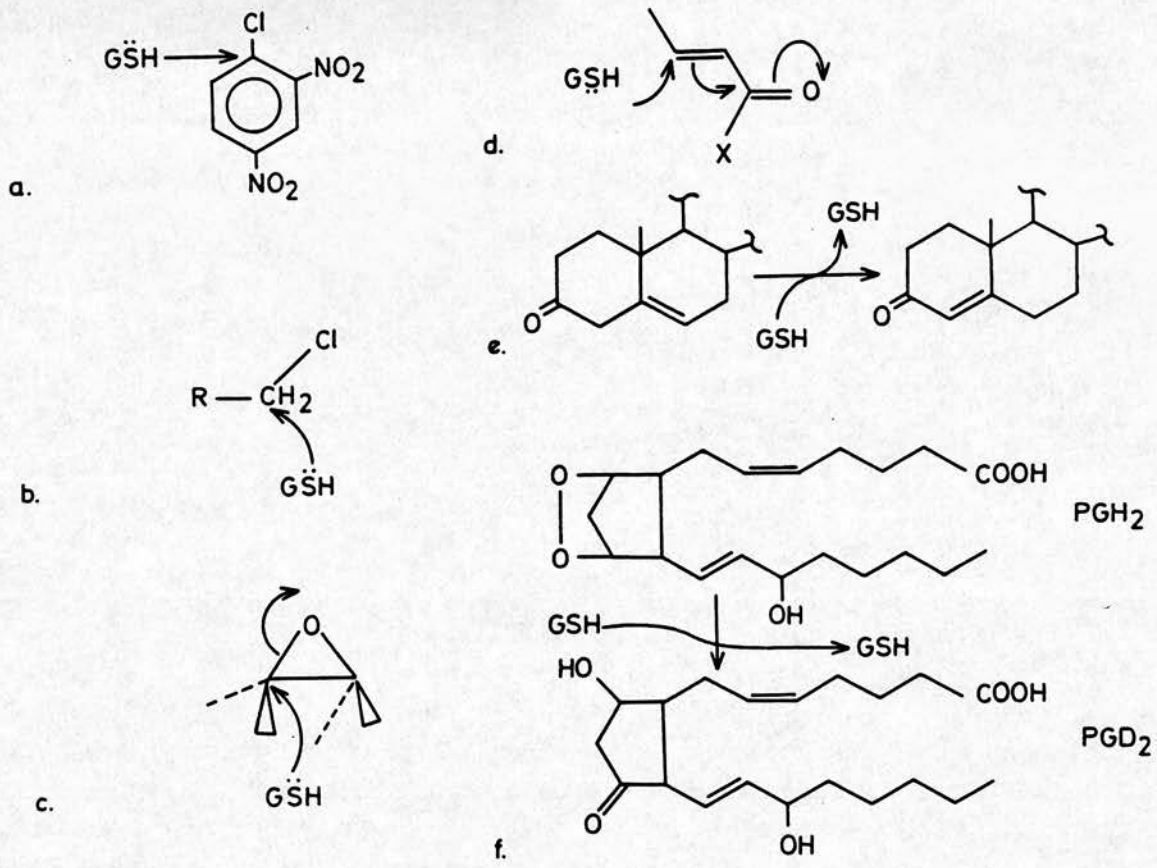

a.
GSH
Cl
NO2
NO2
b.
R — CH2
Cl
GSH
c.
O
GSH
d.
GSH
O
X
e.
GSH
GSH
O
O
f.
O
O
COOH
OH
PGH2
GSH
GSH
HO
COOH
O
OH
PGD2

Figure 1.5.

Figure 1.6. Substrates of GST.

(a) 1,2-Dichloro-4-nitrobenzene

(b) trans-4-Phenyl-3-buten-2-one

(c) 1,2-Epoxy-3-(p-nitrophenoxy)-propane

(d) Ethacrynic acid

(e) p-Nitrophenylacetate

(f) Cumene hydroperoxide

(g) Δ^5-Androstene-3,17-dione

(h) Benzo[a]pyrene-4,5-oxide

(i) 4-Hydroxyalk-2-enal

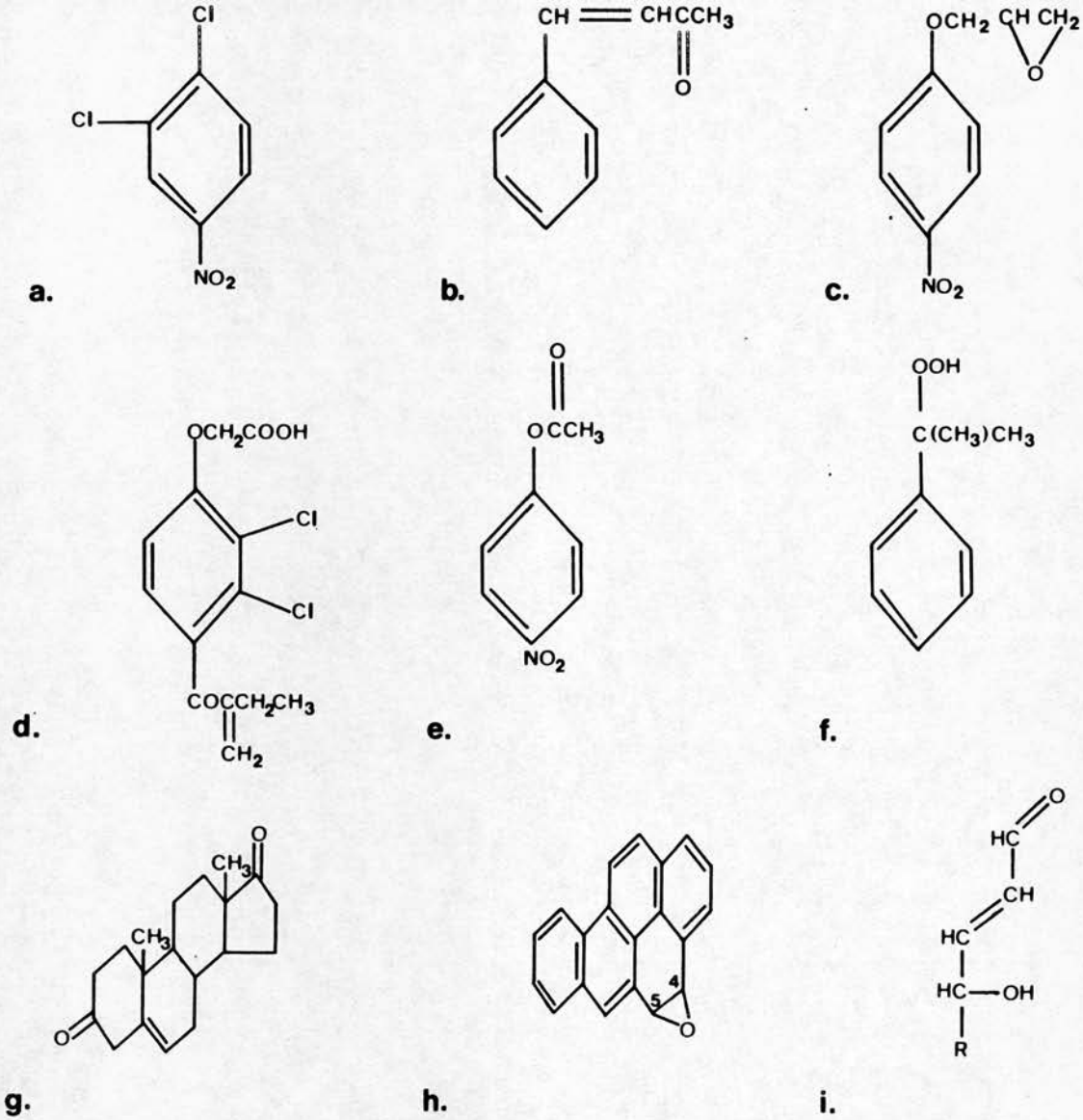


Figure 1.6

1.4 FUNCTION OF GLUTATHIONE S-TRANSFERASE (GST)

a. The physiological role of GST

The physiological importance of the GST enzymes is emphasised by their high concentration (in rat liver, GST accounts for 5% of the cytosolic protein) and their wide distribution throughout the animal kingdom (Jakoby, 1978a; 1978b). The considerable complexity of the mammalian GST system has, in most cases, prevented comprehensive studies of the roles of individual isoenzymes. GST forms occur in many tissues other than liver, being present in significant amounts in testis, kidney, small intestine, heart and lung. These enzymes are subject to a marked tissue-specific expression, but the physiological basis for the tisssue-specific expression is not clear (see section 1.6d).

The GST enzymes are considered to play a multifunctional role in detoxication. They achieve this by acting not only as catalysts but also as binders of non-substrate ligands. Several of these enzymes, notably the Ya-containing forms, have been referred to as ligandin because of their ability to bind a wide spectrum of non-substrate ligands. The physiological roles of ligandin have been reviewed by Arias (1979).

1.4b. Catalytic activity of GST with xenobiotics

GST isoenzymes catalyse the first step in the formation of mercapturic acids and are, therefore, involved in the excretion of potentially harmful electrophilic

compounds from the body (see Fig. 1.1).

Chemical compounds that give rise to mercapturic acids via linkage to GSH are conjugated either directly or after bioactivation such as epoxidation (Boyland & Chasseaud, 1969). To date, *in vitro* studies of GST activity have concerned the use of a very wide range of synthetic substrates, mainly halogenated nitrobenzenes, for reviews see Boyland & Chasseaud, 1969; Benson & Talalay, 1976; Chasseaud, 1976; Keen & Jakoby, 1978; Habig *et al.*, 1974b. However, the question of whether there are endogenous substrates for the GSH transferases in organisms has received surprisingly little attention.

1.4c. Endogenous substrates of GST

The GST family is known to be important in the biosynthesis of endogenous compounds such as the prostaglandins and leukotrienes but it is not yet clear which isoenzymes are responsible *in vivo* for the catalysis. Prostaglandin A_1 is conjugated with GSH by homogenous rat and human transferases (Cagen *et al.*, 1975) and 15-keto-prostaglandins are also found to form conjugates with GSH (Chaudhari *et al.*, 1978). Prostaglandin endoperoxides are converted into a mixture of prostaglandins F_2 , E_2 and D_2 by the action of GST (Fig. 1.5; (Hayakawa *et al.*, 1977).

The GSTs catalyse the conjugation of leukotrienes with glutathione. For example, most of the activity of the slow-

reacting substance of anaphylaxis (SRS-A) can be attributed to leukotrienes C4 and D4. Leukotriene C4 is formed by conjugation of LTA_4 with GSH in the presence of glutathione S-transferase (Fig. 1.7; Hammerstom, 1983). The rat Yb_2 subunit has significant leukotriene C4 synthetase activity (Mannervik et al., 1984) but the major activity, which is found in the microsomal fraction, has yet to be characterized (Bach et al., 1984).

Several other specific compounds which arise in metabolism have been identified as substrates of GST. For example, a GSH-conjugate of dopa (5-S-glutathione-3, 4-dihydroxyphenylalanine) produced in melanocytes may be an intermediate in the incorporation of cysteine sulphur into certain melanins. This conjugate is detected in large amounts in the urine of individuals suffering from malignant melanoma (Agrup et al., 1975, 1977). The formation, in vivo and in vitro, of GSH-conjugates of steroids, suggests that reactive intermediates of estradiol-17β (Jellinck et al., 1967) and 2-hydroxy-estradiol-17β (Elce, 1972) may be substrates for GST. Another group of substrates are the arylalkyl sulphates. Oxidation of the alkyl group of an arylalkyl compound, such as toluene, followed by sulphation, will generate an arylalkyl sulphate (4-nitrobenzyl sulphate). The latter compounds are known to be substrates of glutathione S-transferase M (Gillham, 1971); GST M has yet to be purified and its subunit composition is unknown.

Figure 1.7. Conversion of LTA_4 to LTC_4.

Leukotriene A_4 (LTA_4) is converted to Leukotriene C_4 (LTC_4) by conjugation with GSH. This reaction is catalysed by GST. LTC_4 can be converted into LTD_4 and LTE_4 by successive removal of glutamate and glycine respectively.

Abbreviations: 5-HPETE, 5-hydroperoxy 6, 8, 11, 14 eicosatetraenoic acid.

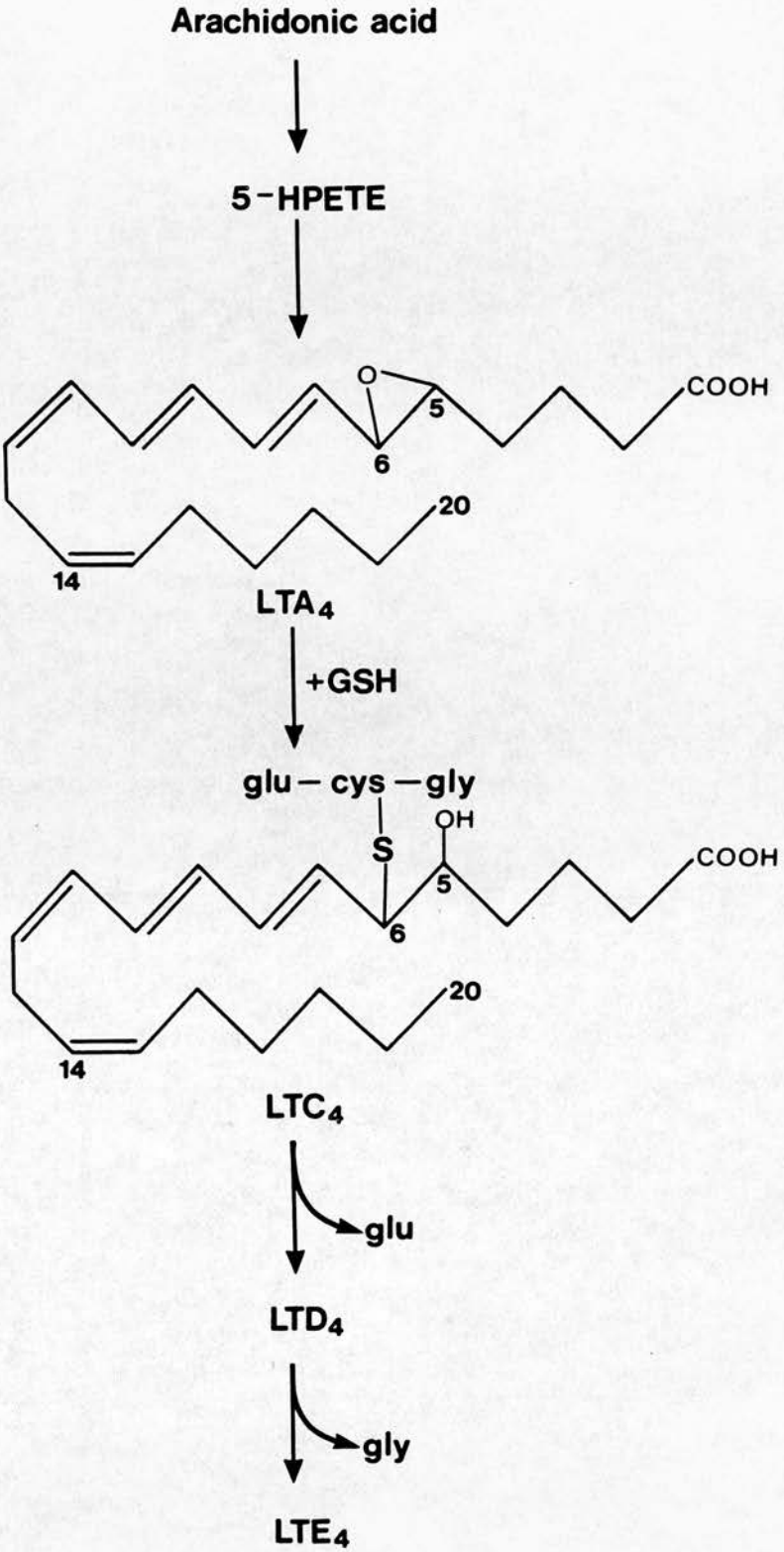

Arachidonic acid
5-HPETE
O
5
COOH
6
20
14
LTA4
+GSH
glu – cys – gly
S
OH
5
COOH
6
20
14
LTC4
glu
LTD4
gly
LTE4

Figure 1.7

It would appear that the GST isoenzymes metabolise a wide spectrum of endogenous compounds, in addition to their role in the detoxification of xenobiotics. Still to be established is the presence of a GST form which is dedicated specifically to the metabolism of an endogenous compound.

1.4d. The role of GST in the elimination of epoxides, hydroperoxides and activated alkenes

Ketterer and his colleagues (Tan et al., 1983) as well as Mannervik (1985) have suggested that an important role of GST is to eliminate the toxic products of oxidative metabolism such as epoxides, hydroperoxides and activated alkenes. Epoxides are produced in vivo by the action of the cytochrome P_{450} enzyme system on both exogenous and endogenous substrates. These compounds are highly reactive and can attack and modify DNA, cause an inhibition in membrane fluidity and inhibit membrane bound enzymes. However, conjugation with glutathione appears to play a role in their elimination. For example, cholesterol α-oxide is a substrate of the rat Ya subunit (Meyer & Ketterer, 1982) and 4-hydroxyalk-2-enals, major products of lipid peroxidation (Esterbauer et al., 1982; Alin et al., 1985a), are substrates for the rat subunit 8 (Yk) (Hayes, 1986; Jensson et al., 1986).

Cytochrome P_{450} can convert environmental pollutants such as polycyclic aromatic hydrocarbons, benzo[a]pyrene and benzanthracene into carcinogens (Fig. 1.8a). The

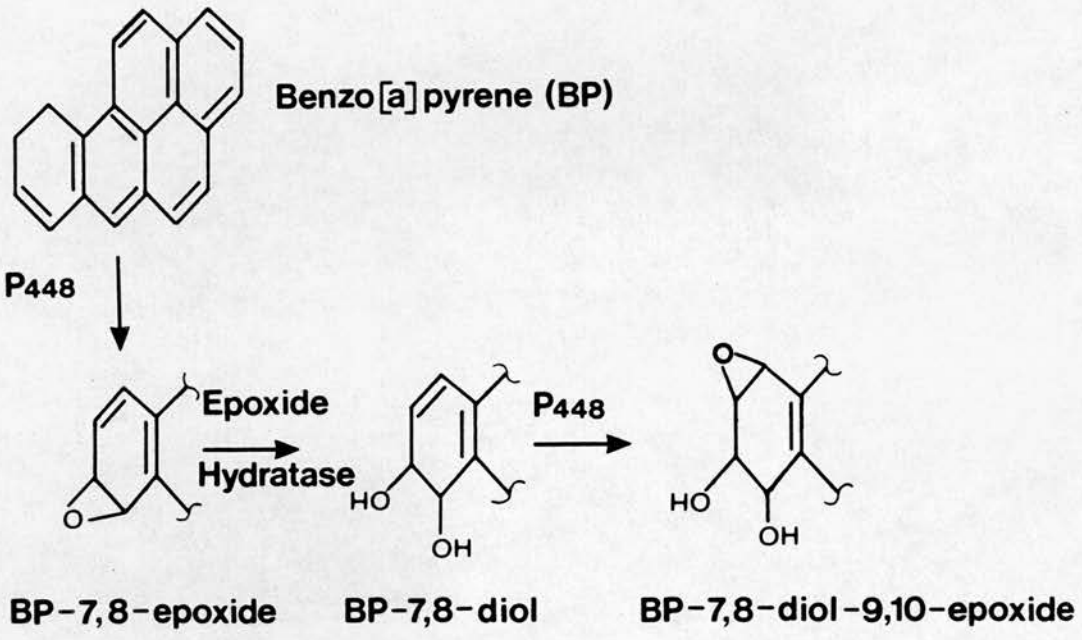
most carcinogenic metabolite of benzo[a]pyrene is the 7,8-diol-9,10 epoxide. It is formed by the action of a special form of cytochrome P_{450}, known as P_{448}, which converts benzo[a]pyrene into a 7,8-epoxide. This epoxide is acted upon by epoxide hydratase to form the 7,8-diol. Epoxidation of benzo[a]pyrene can also occur at other positions but these products are less carcinogenic. All these epoxides are substrates for GST (Glatt et al., 1983) and the conjugates produced are excreted. A fungal product, aflatoxin B_1, obtained from Aspergillus flavus, is converted by cytochrome P_{450} to a series of epoxides including the carcinogen, aflatoxin B_1-8,9-oxide (Fig. 1.8b). These epoxides are substrates for Ya and Yc GST subunits (Coles et al., 1985).

Glutathione S-transferase forms differ widely in their specific activity for epoxide substrates (Warholm et al., 1983). Since a marked polymorphism of these enzymes is observed between individuals, the presence or absence of epoxide metabolising forms may determine the susceptibility of an individual to the actions of chemical mutagens. For example, in humans, transferase μ, which has a high specific activity with benzo[a]pyrene-4,5-oxide, is only present in 60% of the population (Warholm et al., 1983). A microsomal glutathione S-transferase with peroxidase activity has also been implicated in protecting cells from lipid peroxidation (Reddy et al., 1981; Morgenstern & Depierre, 1983).

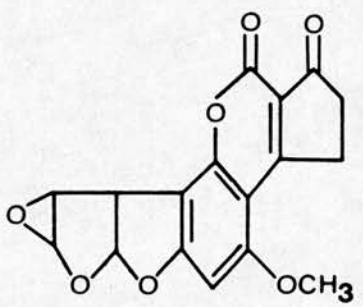
Figure 1.8. Naturally occurring epoxide substrates of GST.

a. Benzo[a]pyrene. Steps in the major pathway of metabolic activation are also shown.

b. Aflatoxin B_1-8,9-oxide.



a.



Aflatoxin B_1-8,9-oxide

b.

Figure 1.8

1.4e. GST as transport and storage proteins

The glutathione S-transferases have been shown to bind a number of lipophilic compounds that are not substrates (Ketley et al., 1975; Tipping et al., 1976). Levi et al. (1969) identified two protein fractions Y and Z in rat liver which were able to bind bilirubin and bromosulphophthalein. The protein fraction referred to as the "Y fraction" was quantitatively the more important of the two (Reyes et al., 1971) because it was present in higher concentrations. The protein in this fraction was subsequently called ligandin (Litwack et al., 1971), because of its ability to bind bilirubin, bromosulphophthalein, cortisol metabolites and azo-dye carcinogen metabolites. Ligandin was considered to be a single protein and was reported to possess a basic isoelectric point and had a molecular mass of about 45 000. Later Listowsky et al. (1976) showed that a preparation of ligandin comprised two nonidentical subunits, now called Ya and Yc (Bass et al., 1977; Hayes et al., 1979). Ligandin possessed similar physicochemical properties to glutathione S-transferase (Kaplowitz et al., 1973) and once purified preparations of GST were available Habig et al. (1974a) reported that ligandin was identical to GST B (YaYc) from rat liver.

Preparations of purified ligandin were described by several groups using different purification techniques (Morey & Litwack, 1969; Habig et al., 1974a; Kamisaka et

al., 1975; Tipping et al., 1976). However, the physico-chemical properties of the various preparations purified by different groups of workers were not identical and suggested that they contained different proteins. Strange et al. (1977) isolated two proteins from the Y fraction which boundlithocholic acid and had GST activity. Hayes et al. (1979) demonstrated that one of these lithocholic acid-binding proteins was composed of YaYa subunits (equivalent to ligandin, purified by the method of Listowsky et al., 1976) and the other comprised YaYc subunits (equivalent to GST B, purified by the method of Habig et al., 1974b). The Ya subunit was later shown to be responsible for the high-affinity binding of lithocholic acid (Hayes et al., 1981). The term ligandin has been used by some workers to include the whole family of glutathione S-transferases (Maruyama et al., 1984; Kamisaka et al., 1975; Ketley et al., 1975) since the binding of non-substrate ligands is not restricted to YaYa protein alone.

Binding of organic anions is a property of most but not all of the rat and human transferases (Kamisaka et al., 1975; Wolkoff, 1980; Maruyama et al., 1984). Ligands for GST include bilirubin and indocyanine green, haem and bromosulphophthalein. The binding of bile salts to glutathione S-transferases has been extensively studied (Tipping et al., 1976; Hayes et al., 1979, 1980; Vessey &

Zakim, 1981; Sugiyama et al., 1984; Hayes & Mantle, 1986a). Binding of glucocortocoids by GST has also been studied (Homma & Listowsky, 1985).

Binding of anions to GST enzymes has been assessed by inhibitory kinetics, circular dichroism, equilibrium dialysis, fluorescence quenching and competitive binding assays. These techniques have yielded a broad range of specific binding constants (Kamisaki et al., 1975; Keen et al., 1976; Tipping et al., 1976) but with considerable lack of agreement.

Inhibition of GST activity (usually assessed using CDNB as substrate) is often produced by the binding of non-substrate ligands such as bile acids (Hayes & Chalmers, 1983). Figure 1.9 shows some examples of known GST inhibitors. The extent of inhibition is pH-dependent (Boyer et al., 1984) and appears to be related to conformational changes of the enzyme-inhibitor complexes. These conformational changes are such that, at high pH the conformers retain their catalytic activity but at low pH they lose catalytic activity (Vander Jagt et al., 1982). Many of these non-substrates, for example, bile acids, haem and bilirubin can occur at physiological concentrations capable of inhibiting the glutathione S--transferase; it is therefore possible that these ligands modify the protective function of GST in vivo.

Figure 1.9. Inhibitors of GST.

a. Cibacron blue. See note below.

b. Lithocholic acid-3-sulphate.

c. Bromosulphophthalein.

d. S-Hexylglutathione.

Cibacron blue. The structure given indicates that the SO_3^- attachment may be in the meta or para position of the "A" ring.

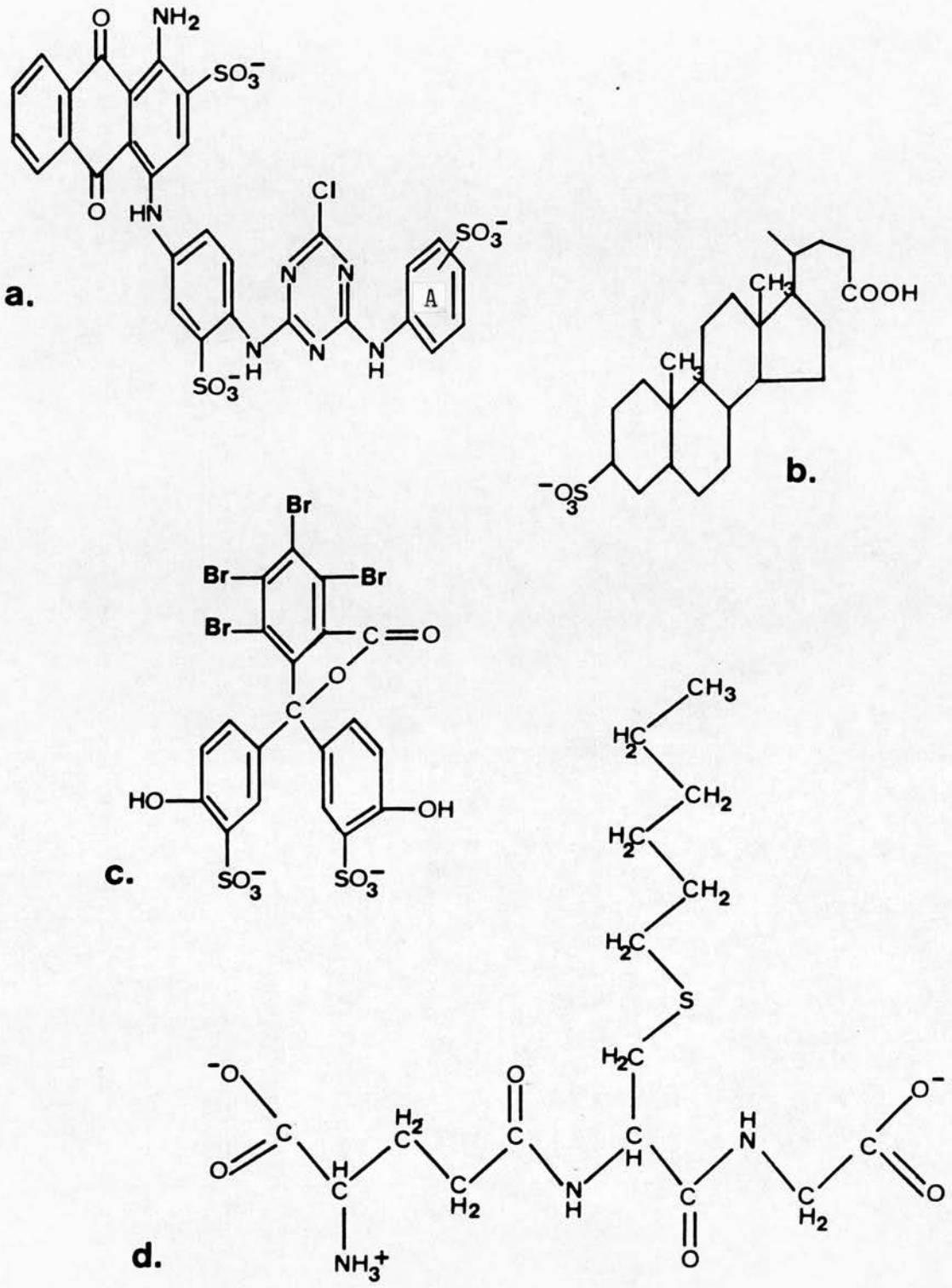


Figure 1.9

A possible role of the glutathione S-transferases may be to act as transport proteins (for a review see Kaplowitz, 1980). This hypothesis is based on the observation that hepatic and renal cells selectively transport compounds that bind to glutathione S-transferases in these cell types (Kaplowitz et al., 1976; Ketterer et al., 1975). Further circumstantial evidence obtained from phylogenetic studies (Levine et al., 1971), investigations into neonatal jaundice (Levi et al., 1970) and induction of ligandin by drugs (Reyes et al., 1971) led to the hypothesis that ligandin is involved in the hepatic uptake of a large number of anions.

It has also been suggested that the glutathione S-transferases may function as intrahepatic storage proteins Wolkoff et al., 1979; Wolkoff, 1980), functioning like an intracellular form of albumin. Many compounds, such as bilirubin, which possess a very limited solubility, could be effectively solubilised by binding to the cytosolic glutathione S-transferases. This would serve to assist the movement of insoluble compounds across the cell or simply create a diffusion gradient for their uptake (Kaplowitz, 1980).

1.4f. Formation of covalent complexes as between GST and reactive electrophiles

Glutathione S-transferases are considered to reduce the susceptibility of the liver to carcinogenesis, not only through their catalytic activity but also by their

ability to bind covalently highly electrophilic compounds. GST protect the nucleus from the mutagenic effects of organic electrophiles (Jakoby & Keen, 1977; Smith et al., 1977). These electrophilic compounds, once bound by glutathione S-transferase, are capable of reacting with glutathione or with a nucleophilic group within the enzyme itself. The latter reaction appears to lead to inactivation of the glutathione S-transferase. In this respect the enzyme sacrifices itself by acting as a target for the electrophile, protecting the other cellular components from damage (Jakoby & Keen, 1977). Examples of this are provided by the work of Ketterer et al. (1967) who isolated an azo-dye carcinogen covalently linked to GST subunits and the work of Yamada & Kaplowitz ((1980) who demonstrated a covalent complex with GST C (Yb_1Yb_2) and ethacrynic acid.

1.5 ENZYMOLOGY OF GLUTATHIONE S-TRANSFERASE (GST)

a. Catalytic mechanism

Little is known about the catalytic mechanism of the majority of GST forms. The first purified enzyme to be studied was transferase A, which is a homodimer comprising Yb_1 subunits.

The steady-state kinetics of GST A from rat liver have been extensively studied. Pabst et al. (1974) demonstrated that the curve obtained by plotting initial reaction rate against substrate concentration for

GST A is non-Michaelian. Two possible explanations could give rise to this behaviour. The first, that the preparations of this enzyme were not homogeneous and secondly, the two identical ligand binding sites of the molecule could interact. However, these explanations were discounted by Jakobson et al. (1979a, 1979b) who demonstrated a hyperbolic binding isotherm for GSH indicating that only one site with an affinity for GSH was present.

The reaction mechanism first proposed was a combination of an ordered sequential with a ping pong mechanism (Pabst et al., 1974). At a GSH concentration of 0.2 mM or below, the electrophilic substrate bound first in a ping-pong mechanism and at higher concentrations an ordered Bi-Bi sequence of addition occurred (for mechanism, see Cleland, 1963).

The intracellular concentration of GSH in rat liver lies between 3 and 8 mmol/l and it therefore appears that the ping-pong kinetics are not of physiological significance. Mannervik and co-workers presented an alternative explanation for the rate behaviour of GST A and suggested a steady-state random sequential mechanism (Askeloff et al., 1975; Jakobson et al., 1977). This model has been supported by stereochemical information from GST catalysed reactions (Mangold & Abdel-Monem, 1980, 1983). These workers demonstrated that GST A catalyses the conjugation

of GSH and phenyl halides with inversion of configuration at the benzylic carbon atom. This result discounts the ping-pong mechanism which operates by double-displacement and retention of the stereochemical configuration. The random sequential mechanism, which includes formation of a ternary complex, involves a single displacement and hence agrees with the experimental findings. Similarly, the use of product analogues of glutathione S-transferase catalysed reactions have been used in double inhibition experiments to support a sequential mechanism for GST A (Jakobson et al., 1979a).

The model reaction mechanism which has been described for GST A, may also be applied to rat liver GST M (Gillham, 1973) and for transferases from the insect larva of Galleria mellonella (Chang et al., 1981) and Costelytra zealandica (Clark et al., 1984).

Any study of the kinetic activity of glutathione S-transferase is complicated by the presence of kinetically stable conformational states which a given enzyme can adopt. The problems associated with this phenomenon were first described by Askelof et al. (1975), who found that pre-incubation of GST A with GSH enhanced the activity of the enzyme. Further examination revealed that certain ligands can cause a time-dependent effect on enzyme activity. Depending upon pH and upon the order with which the reactants are added, the activity of the

enzyme can be altered. Vander Jagt and co-workers have examined the effect of bilirubin binding on the enzyme activity of glutathione S-transferases from human liver (Simons & Vander Jagt, 1980), rat (Vander Jagt, 1982) and human placenta (Vander Jagt et al., 1981). These workers demonstrated that the inhibitory effect of the non-substrate ligand bilirubin on enzyme activity could be modulated by the presence of proteins such as albumin.

1.5b. Active site structure of GST

The active site of glutathione S-transferases has not been investigated using x-ray crystallography nor have active site peptides been isolated. Present-day models of the enzyme active site are based on indirect evidence provided by examination of the requirements of glutathione S-transferases for given substrates and the effects of inhibitors and product analogues. Keen & Jakoby, (1978) proposed that the glutathione S-transferases have two binding sites in close proximity to one another. The first site specifically binds glutathione and the second site binds any one of a large number of hydrophobic electrophiles. This fundamental tenet of this model remains in the active site model proposed by Mannervik (1985). In this, he proposes a GSH binding cavity called the G-site, which has a high specificity for glutathione (Habig et al., 1974b; Kamisaka et al., 1975; Keen et al., 1976). The only other thiol substrate which has been

found to be active with these enzymes is homoglutathione, γ-glutamylcysteinyl- β-alanine (Carnegie, 1963). Adjacent to the G-site is the second substrate binding site, the H-site. The hydrophobic character of this site and its relationship to the G-site have been examined using a series of S-(n-alkyl)-glutathione derivatives (Askelof et al., 1975). These product analogues of the transferases demonstrated an increase in inhibitory strength with increasing chain length, giving support to the concept of a hydrophobic binding domain. Although a wide variety of hydrophobic compounds can bind to this hydrophobic site of these enzymes, each enzyme form demonstrates distinct substrate specificities. This specificity is proposed to arise from the stereochemical requirements for the binding of the scissile bond of the electrophilic substrate adjacent to the sulphydryl group of glutathione bound at the G-site. Active sites occur on each subunit of the enzymes and their catalytic abilities are considered to be independent (Mannervik, 1985).

1.6 THE ISOENZYMES OF RAT GST

a. Multiple forms of GST

Extensive investigations of glutathione S-transferases present in rat liver cytosol have shown that a large number of forms are present (Hayes et al., 1981; Mannervik & Jensson, 1982; Hayes & Chalmers, 1983; Hayes, 1984, 1986). The glutathione S-transferases are dimeric enzymes

and in the rat a series of seven subunit types have been described according to their mobility during SDS/PAGE. These subunits have been listed earlier and are Ya (Mr 25 500), Yb (Mr 26 300), Yc (Mr 27 500), Yf (Mr 24 800), Yk (Mr 25 000) and Yn (Mr 26 000) (Hayes & Mantle, 1986b; Table 1.1). An alternative nomenclature for GST subunits (Table 1.2) has been adopted by some workers. All GST subunit types exhibit broad, overlapping catalytic activities with a wide range of substrates. However, a number of substrates and inhibitors (Table 1.3) are most effective with a particular subunit type and can be used to identify GST forms.

The question of the genetic relationship between the types of glutathione S-transferase subunit in the rat has attracted considerable attention. The elucidation of the number of genes coding these enzymes represents an important and continuing area of research, but one that has proved controversial over the years.

Subunit	Subunit Mr
Yf	24 800
Yk	25 000
Ya	25 500
Yn	26 000
Yb (Yb_1 and Yb_2)	26 300
Yc	27 500

Table 1.1. Apparent sizes of rat GST subunits published by Hayes & Mantle (1986c). SDS/polyacrylamide-gel electrophoresis was performed essentially as described in section 2.2d, using a 12% (w/v) polyacrylamide gel comprising an acrylamide/N,N'-methylenebisacrylamide ratio of 38:1.

Subunit	
Ya	1
Yc	2
Yb_1	3
Yb_2	4
-	5
Yn	6
Yf	7
Yk	8

Table 1.2. Subunit nomenclature of rat GST subunits.

The most commonly used nomenclatures for the GST subunits: column 1, Hayes & Mantle (1986b); column 2, Jakoby et al. (1984).

Subunit	Characteristic Substrate	Characteristic Inhibitor
Ya	Δ^5Androstene-3,17-dione	Hematin
Yb_1	1,2-Dichloro-4-nitrobenzene	Bromosulphophthalein Triethyltin bromide
Yb_2	1,2-Epoxy-3-(p-nitrophenoxy)-propane trans-4-phenyl-3-buten-2-one	
Yc	Cumene hydroperoxide	Tributyltin acetate
Yf	Ethacrynic acid	
Yk	4-Hydroxyalk-2-enals	
Yn	1-Chloro-2,4-dinitrobenzene	Cibacron blue

Table 1.3. Characteristic substrates and inhibitors of rat GST subunits. Data from Mannervik (1985), Mannervik et al. (1985) & Jensson et al. (1986).

1.6b. Families of GST:

Mannervik and co-workers have suggested that the glutathione S-transferases which comprise Yb monomers, those that comprise Ya and/or Yc monomers and those comprising Yf monomers, each represent separate structural groups of enzyme (Mannervik, 1985). Evidence of the different structural origins of the Ya/Yc group of subunits, the Yb group of subunits and the Yf group of subunits is their lack of identity of N-terminal sequences (Frey et al., 1983; Mannervik et al., 1985), the lack of similarity in their peptide maps (Beale et al., 1983; Hayes, 1984) and their immunochemical cross-reactivity (Hayes & Mantle, 1986b).

In vitro subunit hybridisation studies by several groups have shown that subunits from the same, but not from different groups hybridize (Kitahara & Sato, 1981; Boyer et al., 1983; Hayes, 1983, 1984; Kitahara et al., 1983). The GST subunit combinations which occur are presented in Table 1.4. Only certain subunit combinations appear to be possible, thus limiting the extent of GST diversity. Heterodimer formation has been observed between Ya and Yc subunit types and also between Yb and Yn subunit types. However, no YaYb or YbYc heterodimers have been detected. Thus hybrid enzymes containing monomers of different molecular weight can be seen to occur in the Ya/Yc subunit series and in the Yb/Yn subunit series.

The Yf subunit only appears to exist as a homodimer.

Beale and co-workers (Beale et al., 1982) presented the hypothesis that the Ya and Yc subunits are coded for by two separate genes that have arisen by gene duplication and divergence. Hayes (1983) considered the genes coding for the Yb monomers to have arisen by a similar mechanism and suggested that they and the Ya/Yc coding genes are distant relatives of a common ancestral gene. These ideas are echoed in the hypothetical scheme for the evolution of glutathione S-transferases presented by Mannervik (1985).

Subunit combination	Trivial name	Reference
YaYa	ligandin	Hayes et al., 1979
YaYc	B	Scully & Mantle, 1981
YcYc	AA	Hayes & Clarkson, 1982
Yb_1Yb_1	A	Hayes, 1983; Beale et al., 1983
Yb_1Yb_2	C	Hayes, 1983; Beale et al., 1983
Yb_2Yb_2	D	Hayes, 1983; Beale et al., 1983
Yb_1Yn	P*	Hayes, 1984
Yb_2Yn	S	Hayes & Chalmers, 1983
YnYn	N	Hayes, 1984
YfYf		Hayes, 1984
YkYk	K	Hayes, 1986

Table 1.4. Combinations of rat GST subunits.

*Note that the GST-P described by Hayes is a YbYn heterodimer and should not be confused with GST-P, a YfYf homodimer described by Satoh et al. (1985).

Nucleotide sequence analysis of GST cDNA has revealed a genetic basis for subunit diversity. It appears that subunit groups Ya/Yc, Yb/Yn and Yf have distinct genetic origins and are not modified forms of a single gene. Comparisons of full length cDNA sequences and partial peptide sequences of Ya, Yb, Yc and Yf subunits suggest that they are the products of distinct gene families which have evolved independently (Beale et al., 1982; Tu et al., 1982b; Hayes, 1983; Lai et al., 1984; Pickett et al., 1984; Alin et al., 1985b; Ding et al., 1985; Mannervik et al., 1985; Sugioka et al., 1985; Telakowski-Hopkins et al., 1985; Ding et al., 1986). Rat liver glutathione S-transferase Ya and Yc mRNAs possess 75% nucleotide sequence identity in the protein coding region indicating that they are encoded by closely related, but distinct genes. The amino acid sequence homology of the Ya and Yc subunits is 68%. Interestingly, significant sequence divergence exists in the 5' and 3' untranslated regions of the Ya and Yc mRNAs (Lai et al., 1984; Pickett et al., 1984). By contrast, the Yb subunit type appears to be distantly related to the Ya and Yc subunits. The Ya and Yc mRNAs possess little nucleotide sequence homology with the Yb_1 and Yb_2 mRNAs and the Yb_1 and Yb_2 subunits exhibit only 25% amino acid sequence homology with the Ya and Yc subunits (Ding et al., 1986).

A full length cDNA clone has been obtained for the Yf subunit of GST-P. This subunit also appears to be the product of a distinct gene which may have evolved before the Ya and Yc genes diverged. The Yf has only 32% amino acid homology with the Ya subunit (Sugioka et al., 1985). The relationship between the Yn and Yk subunits and the other subunit types, remains to be established.

1.6c. Multiple copies of individual subunit types

At least six GST subunit types have been described, based on their mobility on SDS/PAGE. There is now considerable evidence to demonstrate that each of these GST polypeptides exists in more than one form. To date, multiple forms of Ya and Yb subunits have been demonstrated.

The YaYa homodimer described by Hayes & Clarkson (1982) and transferase X described earlier, (Hales et al., 1978; Scully & Mantle, 1981) were initially considered to be the same protein. However, Sheehan & Mantle (1984) isolated two kinetically distinct forms of GST YaYa. Furthermore, two groups (Lai et al., 1984; Pickett et al., 1984) have obtained full-length cDNA clones for the Ya subunit and several other groups of workers (Tu et al., 1982a; Kalinyak & Taylor, 1982; Daniel et al., 1983; Taylor et al., 1984) have obtained partial cDNA sequences of Ya subunits. The two full length clones, pGTR 261 (Lai et al., 1984) and pGTB 38 (Pickett et al., 1984)

differ in sixteen nucleotide positions or nine amino acids in the final polypeptide. A comparison of the full and partial cDNA structures suggests that at least two polypeptide sequences can be assigned to the Ya monomer.

At least two distinct Yb type subunits exist in the rat. Mannervik and co-workers demonstrated two Yb monomers of Mr 26 300 (Yb_1 and Yb_2) could be distinguished on the basis of substrate specificity and differential inhibition by a number of inhibitors (Mannervik & Jensson, 1982; Yalcin et al., 1983; Mannervik, 1985). The N-terminal amino acid sequences (Frey et al., 1983) and peptide maps (Beale et al., 1983; Hayes, 1983) of Yb_1 and Yb_2 indicated differences in their primary structure. This was confirmed when cDNA clones pGTA/C44 (Ding et al., 1985) and pGTA/C48 (Ding et al., 1986) were obtained for the Yb_1 and Yb_2 subunits respectively. A comparison of the nucleotide sequences revealed that in the protein coding region the sequences were 84% homologous. In the 3´ untranslated regions, the Yb_1 and Yb_2 clones were only 32% homologous. These data indicate that the Yb_1 and Yb_2 subunits arise from two distinct but closely related genes.

In conclusion, it would appear from nucleotide sequence data that GST in the rat is coded for by at least three supergene families. The Ya and Yc subunits are the products of one gene family, the Yb_1 and Yb_2 subunits

are the products of another gene family and the Yf subunits are the products of yet another gene family.

1.6d. Expression of GST subunits in extrahepatic tissues

In the rat, glutathione S-transferases are subject to a marked tissue-specific expression (Table 1.5). Not every tissue expresses all possible forms of GST. For example, YfYf is not significantly expressed in normal rat liver but is a predominant form in many other tissues. Regional variations in GST distribution in the intestine are apparent, the Ya subunit is a major form in the small intestine but not in the colon.

It is believed that the amount of a GST subunit expressed in a tissue is determined by the level of transcriptional activation of the gene (Ding & Pickett, 1985; Sugioka et al., 1985; Ding et al., 1986; Li et al., 1986). However, the factors which regulate this process have yet to be identified.

1.6e. Multiple forms of GST arising as purification artefacts

Several post-synthetically modified glutathione S-transferases have been demonstrated to occur during purification. This phenomenon often alters their elution characteristics generating multiple peaks of activity. Consequently multiple peaks of activity eluted on column chromatography are not indicative of multiple isoenzyme forms (Hayes & Clarkson, 1982; Friedberg et al., 1983; Hayes, 1984). For example, the Yb_1Yb_1 dimer exists in

Intensity of peroxidase staining

Organ Subunit	Liver (4)	Kidney (1.1)	Lung (0.9)	Heart (0.4)	Spleen (0.5)	Testis (4.4)	Thymus (0.7)	Small Intestine (1.6)	Colon (2.0)
Yc	+++	+++++	++++	++	++++	++++	++++	Trace	Trace
Yb_1	++++	Trace	++	+++	++	+++	+++	++	++++
Yb_2	++++	+	++	+++	+	+++	++	++	++++
Yn	Trace	-	-	++	+	+++++	+	-	-
Ya	++++	++++	Trace	Trace	Trace	Trace	Trace	++++++	Trace
Yk	+	++	+++	++	++	+	+	++	++
Yf	Trace	++++	+++++	++++	++++++	Trace	+++++	+++	++++++

Table 1.5. Tissue distribution of GST in the rat.

Data originally published by Hayes & Mantle (1986b). Relative amounts of GST were determined by 'Western' blotting analysis of GST forms, obtained from tissue cytosol by S-hexylglutathione affinity chromatography. Numbers in brackets are % of total cytosolic protein accounted for by GST.

three forms (A_1, A_2 and A_3) which are thought to arise as the result of autoxidation of a single residue in the Yb_1 polypeptide (Hayes & Clarkson, 1982; Friedberg et al., 1983). Mixed disulphide formation between glutathione S-transferases and glutathione, used for affinity chromatography, was thought to generate charge isomers of the enzymes (Ramage & Nimmo, 1983).

The binding of non-substrate ligands to glutathione S-transferases is known to cause alterations in the elution characteristics of these enzymes. Hayes (1984) has noted an important example of this phenomenon concerning the ligand, S-hexylglutathione, which was used for the affinity purification of GST YnYn (GST N). The author suggested that an interaction between S-hexylglutathione and GST YnYn resulted in this enzyme being resolved into three forms. However, prolonged dialysis of the affinity purified GST YnYn preparation, to remove S-hexylglutathione, resulted in the elution of only a single GST YnYn form on subsequent chromatofocusing. Modification of other forms of glutathione S-transferase by this widely used technique has not been described. However, since many of the glutathione S-tranferases bind S-hexyl-glutathione, it is potentially a widespread phenomenon.

1.7 THE ISOENZYMES OF HUMAN GST

a. An overview of the GST forms in man

In human tissues, as in tissues from other species, different isoenzymes of glutathione transferases are present.

The multiple forms of human transferases can be divided according to their charge into three groups; the basic, the neutral and the acidic enzymes (Warholm *et al.*, 1983). The relationship between the enzymes, within a group and between groups has been a source of controversy.

Although detailed information is available about the structure and function of GST in the rat, relatively little is known about these enzymes in man. The first report of the purification and characterisation of human glutathione S-transferases demonstrated that they too were basic dimeric enzymes of subunit Mr 25 000 capable of conjugating glutathione with a wide range of electrophiles (Kamisaka *et al.*, 1975).

1.7b. Basic GST

Five basic transferases $\alpha, \beta, \gamma, \delta$ and ϵ were first isolated and characterised from a single human liver. Kamisaka *et al.* (1975) applied human liver extract to DEAE-cellulose, and the activity that was eluted in the flow-through fractions was resolved by CM-cellulose chromatography. Transferases α, β and γ did not bind the CM-cellulose cation exchanger; these were resolved by chromatography on hydroxyapatite and column isoelectric

focusing. By contrast, transferases δ and ϵ were eluted sequentially from CM-cellulose by a KCl gradient. Transferases α - γ were reported to have pI values of 7.8 - 8.55, whereas transferases δ and ϵ were shown to have pI values of 8.75 and 8.8 respectively. Amino acid analyses indicated the five transferases to be either very closely related or identical in this respect. The enzymes had molecular weight of 48 500 consisting of apparently identical subunits.

Each of the human transferases had a similar specific activity for a given electrophilic substrate and reacted with the gamma globulin fraction of a goat antibody to human ligandin using Ouchterlony immunodiffusion (Kamisaka et al., 1975). The amino-acid composition and catalytic properties of the enzymes as well as their immunological identity led the authors to suggest that they were charge isomers of a single gene product. The mechanism which generated the isomerisation was not established but deamidation in vivo was considered a likely possibility. Unfortunately, differences in the amide nitrogen content between each of the enzyme forms were not demonstrated due to shortage of material.

Analytical gel electrofocusing of the purified enzymes α, β and ϵ gave single focused bands of activity. However, forms α and β were poorly resolved by this technique. Transferases γ and δ migrated as two and

three bands respectively and in the latter case interconversion from one enzyme form to another was observed by Kamisaka et al. (1975). Since the enzymes were stored in the presence of exogenous mercaptan, the authors proposed that this interconversion was mediated by disulphide formation or interchange, but this was not investigated further.

To investigate if the five enzyme forms were artefacts of the purification procedure, a cell-free extract of a small fresh portion of the same liver as that used for the purification of these enzymes, was prepared and subjected to gel electrofocusing in the range pH 7-10. From the published activity profile of this gel, the authors concluded that the numbersof active fractions resolved were consistent with those obtained during enzyme purification. Thus Kamisaka et al. (1975) concluded, that multiple human hepatic GST forms exist in vivo and GST α-ϵ were not generated during the purification procedure.

1.7c. Acidic GST

Glutathione S-transferases with acidic isoelectric points have been demonstrated to occur in human tissues, the first to be described being from erythrocytes (Marcus et al., 1978). Later, a similar enzyme was isolated from the placenta (Guthenberg et al., 1979). These acidic enzymes had many features in common and were demonstrated to be quite distinct from the basic enzymes in human liver. In addition to their acidic isoelectric points (pH

4.6-4.9) they had molecular weights (Mr 46 000) and immunochemical properties that differed from those determined for the basic enzymes. Koskelo (1983) partially purified the major acidic transferases from several human organs: kidney, lung, spleen and placenta and concluded that they are either closely related or identical, a view widely accepted by workers in this field (Mannervik, 1985). A similar acidic GST has also been purified from human fetal liver (Warholm et al., 1981b). However, Awasthi and co-workers (Partridge et al., 1984) suggested that the lung and placental, acidic enzymes are closely related and distinct from both the liver and the erythrocyte acidic GST. This view is endorsed by Tahir et al. (1985) who referred to unpublished work demonstrating the use of inhibitors to differentiate between these groups.

Two forms of acidic GST are reported to occur in adult human liver. While the basic enzymes represent most of the glutathione S-transferase activity in this tissue, a small proportion (10%) can be attributed to acidic GST (Awasthi et al., 1980; Koskelo & Valmet, 1980; Warholm et al., 1980). These acidic enzymes, referred to as ω and ψ, have been purified and partially characterised by Awasthi et al. (1980) who reported that these forms are immunologically related to the basic enzymes.

1.7d. Neutral GST

A transferase with a neutral isoelectric point has been demonstrated to occur in the population with a fre-

quency of approximately 60% (Warholm et al., 1980). This enzyme, GST μ, has been purified from human liver (Warholm et al., 1981a). Transferase μ is a dimer of subunits Mr 26 300. This and other physicochemical properties suggested that GST μ is separate from the basic or acidic enzymes (Warholm et al., 1983).

GST μ is notable for its high activity with the substrates trans-4-phenyl-3-buten-2-one and benzo[a]pyrene -4,5-oxide (Warholm et al., 1981a; 1983). This activity with the latter substrate suggested that individuals who express the enzyme are conferred with a better protection against some chemical mutagens and carcinogens than those who do not (Warholm et al., 1983). Individuals who demonstrate no GST μ enzymic activity, appear to be unable to produce any GST μ polypeptide (Mannervik, 1985). It is thought likely that failure to produce the active neutral glutathione S-transferase occurs at the level of gene expression.

1.7e. Classification of human GST

The rationale of the original classification of human glutathione S-transferases was based on differences in the isoelectric points of the individual enzymes. Using this method the enzymes could be assigned to one of the three groups, basics, neutrals or acidics (Warholm et al., 1983). Mannervik and co-workers have noted that the catalytic properties of the enzymes within each of these groups are very similar but distinct from those of the

other groups. This provided a functional basis for the original classification and these workers demonstrated that a selected number of substrates (Warholm et al., 1983) or specific inhibitors (Tahir et al., 1985) could be used to distinguish the three types of human glutathione S-transferase. Hence, the basic group is characterised by high glutathione peroxidase activity with organic hydroperoxides (Awasthi et al., 1980; Warholm et al., 1983) and high steroid isomerase activity with Δ^5-androstene3,17-dione (Warholm et al., 1983). The neutral group is highly active with trans-4-phenyl-3-buten-2-one, styrene-7,8-oxide and benzo[a]pyrene-4,5-oxide (Warholm et al., 1983). Finally, the acidic transferases are highly active with ethacrynic acid (Marcus et al., 1978; Guthenberg & Mannervik, 1981).

Alternatively, various compounds can be used to inhibit the standard assay with 1-chloro-2,4-dinitrobenzene as electrophilic substrate. For example, Tributyltin acetate is the most potent inhibitor of the basic transferases and Cibacron blue is the most effective inhibitor with the near-neutral and the acidic types. While no compound was found to be more effective at inhibiting the acidic transferases compared to the basic or neutral forms, the use of both these inhibitors permitted all three types of human enzyme to be distinguished (Tahir et al., 1985).

Indirect evidence of structural similarities between proteins can be obtained by examining their immunochemical properties. This approach has been used to distiguish between isoenzymes of rat glutathione S-transferase (Jakoby, 1978a; Mannervik & Jensson, 1982; Hayes and Mantle, 1986b). However, in the human there is a lack of agreement concerning the cross-reactivity data for these enzymes. While Mannervik and co-workers have reported that the basic, neutral and acidic groups of enzymes are immunochemically distinct (Warholm et al., 1983), other workers have demonstrated that enzymes from the basic and acidic groups share common antigenic determinants (Awasthi et al., 1980; Dao et al., 1982). Mannervik and colleagues separately raised antisera against a combined pool of basic enzymes (α - ϵ), the neutral enzyme (μ) and the acidic placental enzyme (π). Each of these antisera was reported to react with their corresponding immunogens but not with the other transferases (Warholm et al., 1983). These workers demonstrated that the antiserum raised to the acidic placental enzyme, cross-reacted with other acidic transferases from the same group i.e. erythrocyte glutathione S-transferase (ρ) and the fetal acidic enzyme (Guthenberg & Mannervik, 1981; Warholm et al., 1983). Thus Mannervik and his colleagues reported that antisera raised to GST from one group of enzymes do not cross-react with GST forms from other groups.

Awasthi et al., (1980) also raised an antiserum against the combined liver basic enzymes. However, by contrast with the data of Warholm et al. (1983), Awasthi et al. (1980) reported that their anti-basic GST-IgG cross-reacted with the acidic GST (ω) isolated from adult liver. In this case Awasthi et al. (1980) argued that a structural inter-relationship exists between the enzymes from the basic and acidic groups.

1.7f. Subunit composition of human GST

In comparison with the rat glutathione S-transferases very little is known about the structure of the human enzymes. Although most of the available information supports the hypothesis that there are three distinct groups, there are suggestions that the transferases in man are coded for by only one gene and that multiple forms arise as the result of post-synthetic modifications.

It is widely agreed that all human glutathione S-transferases are dimers composed of subunits of equal molecular weight (Mannervik, 1985). Estimates of the values for these molecular weights vary, but it is clear that three classes of subunits occur, based on their electrophoretic mobility. The subunits of the acidic and neutral enzymes exhibit the fastest and slowest mobilites respectively on SDS/PAGE, while those of the basic enzymes have an intermediate mobility (Hayes et al., 1983; Warholm et al., 1983; Mannervik, 1985). However, Awasthi and co-

workers report that the basic liver transferases and the acidic transferase are heterodimers. The basic and acidic enzymes are considered by Awasthi and his colleagues to share a common subunit of Mr 24 500 which combines with additional subunits Mr 26 500 and Mr 22 500 to form basic and acidic dimers respectively (Dao et al., 1982; Awasthi & Singh, 1985).

Contrary to these results, the same authors have identified the basic liver transferases (α - ϵ) and the acidic GST,ω as homodimers with subunits Mr 24 500 and Mr 22 000 respectively (Awasthi et al., 1980). The significance of these different observations have not been discussed.

1.7g. Primary structure of human GST

The value of primary structural information has been realised for rat liver glutathione S-transferases. Amino acid sequence data, derived from full and partial cDNA structures of the GST subunits, have revealed their underlying genetic differences (Mannervik, 1985). However, sequence information concerning the human glutathione S-transferases is much less comprehensive. Structural differences in the N-termini of glutathione S-transferases from each of the three groups has been presented as evidence of their different genetic origins (Alin et al., 1985b). A 23 residue N-terminal amino acid sequence of neutral GST (μ) was demonstrated to be significantly different from the same region of the acidic pla-

cental transferase (π). In addition, the pooled group of basic liver glutathione S-transferases (α - ϵ) were found to have blocked N-termini which prevented further sequence analysis.

The N-terminal amino acid sequence of placental glutathione S-transferase originally reported by Dao et al. (1984) is identical to that of the acidic enzyme from human lung (λ) (Alin et al., 1985b).

Grover (1982) has proposed that limited proteolysis is responsible for generating the multiple forms of the human enzymes. The amino acid compositions of these glutathione S-transferases (Kamisaka et al., 1975; Marcus et al., 1978; Awasthi et al., 1980; Guthenberg & Mannervik, 1981; Pattinson, 1981; Warholm et al., 1983; Partridge et al., 1984; Alin et al., 1985b) demonstrated significant differences between the enzymes from each of the three groups. However, none of the amino acid compositions was consistent with the possibility that the proteins could be derived from each other by limited proteolysis (Alin et al., 1985b).

1.8 GENETIC REGULATION OF HUMAN GST
Polymorphism and tissue distribution

The first model of genetic regulation to be proposed for the glutathione S-transferases in humans was presented by (Board 1981a, 1981b). He studied the genetic variation of glutathione S-transferases in the human population using

starch-gel electrophoresis and concluded that these enzymes were the products of three structural gene loci: GST 1, GST 2 and GST 3. Board (1981a) demonstrated that when liver cytosol was subjected to starch-gel electrophoresis at pH 8.6, multiple bands of glutathione S-transferase activity were revealed, with CDNB as substrate. These zones of activity migrated towards both anode and cathode. The strongly staining anodal components were proposed to be the product of one locus, GST 1. The GST 1 zone was not always present in tissue samples. The strongly staining cathodal components were the products of the GST 2 locus and weakly staining, very-fast-migrating, anodal components, were the products of the GST 3 locus. Both the GST 1 and GST 2 components exhibited triplet patterns, characteristic of those obtained from heterozygotes for a polymorphic dimeric protein (Board 1981a). The populations sampled appeared to achieve Hardy-Weinberg equilibrium if it was assumed that two alleles were present at the GST 2 locus, one allele at the GST 3 locus and three alleles at the GST 1 locus, one of which was nulled i.e., not expressed. Although it had previously been suggested that the multiple forms of glutathione S-transferase were the products of post-translational modification of a single gene product (Kamisaka et al., 1975), the data of Board (1981a, 1981b) presented an alternative, genetic interpretation to their

origin. Later work by Strange et al. (1984), using the same technique to investigate the tissue distribution of human glutathione S-transferases, proposed that these enzymes arose from the three gene loci described by Board (1981a, 1981b) but the products of the GST 2 locus were subject to post-translational modification.

1.9 AIMS OF THE THESIS

The aims of the thesis were to; (1) purify the multiple forms of cytosolic glutathione S-transferase which occur in human liver; (2) determine the catalytic and physicochemical properties of these enzymes; (3) determine the relationship of these enzymes with the glutathione S-transferases which had been previously described; (4) identify if hybrid enzymes exist; (5) define the structural origins of the multiple forms of basic enzyme.

Experiments were performed to determine the amino acid compositions, isoelectric points and subunit molecular weights of purified enzyme forms. The catalytic activities of the enzymes were investigated using a range of specific substrates and inhibitors. Immunoblotting and peptide 'mapping' of transferases were performed to investigate their immunological and structural identities respectively.

SECTION 2: MATERIALS AND METHODS

MATERIALS

2.1 CHEMICALS

a. Suppliers

Aldrich Chemical Co., Gillingham, Dorset, U.K.

trans-4-Phenyl-3-buten-2-one.

B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

Acetone; acetic acid; acrylamide; 2-amino-2-(hydroxymethyl) propane-1,3-diol (Tris); ammonium persulphate; bromophenol blue; bromosulphophthalein; butan-2-ol; chromium trioxide; 1-chloro-2,4-dinitrobenzene; cumene hydroperoxide; dipotassium hydrogen orthophosphate; sodium hydrogen orthophosphate; ethanol; hematin; hydrochloric acid; hydrogen peroxide; 2-mercaptoethanol; methanol; NN´-methylenebisacrylamide; orthophosphoric acid; potassium dihydrogen orthophosphate; sodium chloride; sodium dihydrogen orthophosphate; sodium dodecyl sulphate; sulphuric acid; NNN'N' -tetramethyl ethylenediamine; trichloroacetic acid; urea.

Bio-Rad Laboratories, Watford, Herts, U.K.

Nitrocellulose paper and immuno-blot assay kit.

Chromatography Services Ltd., Hoylake, Wirral, Merseyside, U.K.

CM-cellulose (Whatman CM52) and DEAE-cellulose (Whatman DE52).

Kodak Limited, Kirkby, Liverpool, U.K.

Tributyltin acetate; triethyltin bromide; triphenyltin chloride.

LKB-Products, Bromma, Sweden

Ampholine solutions.

Millipore (U.K.) Limited, Harrow, Middlesex, U.K.

Membranes for ultrafiltration cell.

Pharmacia Fine Chemicals, Uppsala, Sweden

Protein standards for isoelectric focusing.

Scientific Instrument Centre Ltd., London, U.K.

Visking dialysis tubing (molecular cut-off approximately Mr 10 000).

Scottish Antibody Production Unit, Carluke, Lanarkshire, Scotland, U.K.

Normal rabbit serum and donkey anti-rabbit serum.

Serva Feinbiochemica, Heidelberg, West Germany

Coomassie blue G (Serva blau G).

Sigma Chemical Co. (London) Ltd., Poole, Dorset, U.K.

Bilirubin; bovine serum albumin; 1-chloro-2,4-dinitrobenzene Cibacron blue 3GA; cholic acid; Coomassie brilliant blue R; DEAE-Sephadex A-50; 1,2-dichloro-4-nitrobenzene 1,2-epoxy3-(p-nitrophenoxy)-propane; ethacrynic acid; ethanolamine; Freund's adjuvant (complete and incomplete); glutathione (reduced form); glutathione reductase; lithocholic acid-3-sulphate; nicotinamide adenine dinucleotide phosphate; p-nitrobenzyl chloride; p-nitrophenethyl bromide; Pharmalyte pH 8-10.5; Polybuffer 96; Polybuffer 74; Polybuffer exchanger 118; Polybuffer exchanger 94; Sephadex G-25 fine; Sephadex G-100 - 120; triethylamine; trypsin.

Sigma Chemical Co., (contd.)

L-amino guanidinopropionic acid, L-norleucine and the standards for high-voltage paper electrophoresis ('Wonder mix', which comprised lysine, histidine, arginine, glycine, valine, alanyl glycine, ϵ-N-dinitrophenyl lysine, taurine, cysteic acid, aspartic acid, glutamic acid and xylene cyanol FF) were a gift from Professor R P Ambler, Department of Molecular Biology, University of Edinburgh, Edinburgh.

2.1b. Synthesis of S-hexylglutathione

S-Hexylglutathione was synthesised by coupling iodohexane to reduced glutathione via the cysteine sulphydryl group as described by Vince et al. (1971). The S-hexylglutathione affinity matrix was made by coupling the γ-glutamyl moiety of S-hexylglutathione to epoxy-activated Sepharose 6B as described by Mannervik & Guthenberg (1981).

2.1c. Tissue

Human liver was obtained between 4 h and 24 h post mortem from individuals who had died from a disease that had not involved the liver. The livers were stored at -70°C until required.

2.1d. Antisera and protein standards

Antisera and protein standards used for radioimmunoassay of GST B_1B_1 and GST B_2B_2 were kindly supplied by Dr G J Beckett and Dr J D Hayes. Rat GST Ya, Yb and Yc

and human lung GST (λ), used as molecular weight standards for SDS/polyacrylamide-gel electrophoresis, were also a gift from Dr J D Hayes.

2.2 GENERAL ANALYTICAL METHODS

a. GST enzymatic assays

Glutathione S-transferase activities with various electrophilic substrates were determined at 37°C essentially according to the procedures described by Habig et al. (1974b), Lawrence & Burke (1976), Keen & Jakoby (1978), and Habig & Jakoby (1981) as outlined in Table 2.1. All assays were carried out in a spectrophotometer, except those for the substrates 1,2-dichloro-4-nitrobenzene (DCNB) and 1-chloro-2,4-dinitrobenzene (CDNB).

GST activity with either CDNB or DCNB was measured at 37°C by using a centrifugal analyser (Rotochem IIa parallel fast analyser, American Instrument Co., Silver Spring, Md, USA). The assay for measuring the conjugation of both CDNB (1 mmol/litre) with GSH (2 mmol/litre) and DCNB (1 mmol/litre) with GSH (5 mmol/litre) was carried out in 100 mM-sodium phosphate buffer at pH 6.5 and 7.5 respectively. The samples were preincubated with GSH and the reactions (a maximum of 35 were analysed simultaneously) were initiated by centrifugation. Reactions were monitored by measuring the absorbance at 340 nm at 10s after mixing and subsequently on nine occasions thereafter at 5s intervals. Reaction rates were

Substrate	[Substrate] (mM)	[GSH] (mM)	pH	Extinction Coefficient (mM^{-1} cm^{-1})	Wavelength (nm)
1-Chloro-2,4-dinitrobenzene[a]	1.0	2.0	6.5	9.6	340
1,2-Dichloro-4-nitrobenzene[a]	1.0	5.0	7.5	8.5	345
p-Nitrophenyl acetate[b]	0.2	0.5	7.0	8.8	400
1,2-Epoxy-3-(p-nitrophenoxy)-propane[a]	0.5	5.0	6.5	0.5	360
trans-4-Phenyl-3-buten-2-one[a]	0.05	0.25	6.5	-24.8	290
Ethacrynic acid[a]	0.2	0.25	6.5	5.0	270
Cumene hydroperoxide[c]	1.5	1.0	7.0	- 6.2	340

Table 2.1. Conditions for spectrophotometric assays of GST activities. These were carried out in a thermostated cell compartment at 37°C according to the methods of (a) Habig & Jakoby, 1981; (b) Keen & Jakoby, 1978 and (c) Lawrence & Burke, 1976.

Assays in which the absorbance decreases during the conjugation of substrate with GSH are indicated by a negative value for the extinction coefficient.

Determination of multiplication factor for CDNB assay

$$U = \frac{R \times v}{E}$$

where:

U = Enzyme Units per ml (U/ml), (μmol/ml/min).

R = Change in absorbance (ΔA_{340}/min/ml).

v = Reaction volume (ml).

E = Extinction coefficient ($mM^{-1}cm^{-1}$).

for CDNB assay v = 0.5 ml

E = 9.6 $mM^{-1}cm^{-1}$.

$\therefore$ U = R x 0.052.

determined by using the manufacturer's kinetic rate program to fit regression curves to the nine A_{340} values that were obtained from each cuvette. Values for the reaction rate were obtained in units , ΔA_{340}/min/ml,(R).A multiplication factor of 0.052 is required to convert these to Enzyme Units per ml (U/ml), (μmol/ml/min), for CDNB assay.[1]

The effects of inhibitors on the activity of GST were investigated, using CDNB as substrate, essentially according to the procedures described by Tahir et al. (1985). Stock solutions of each inhibitor were prepared and included at the appropriate dilution in the assay buffer.

2.2b. Protein determination

Protein concentrations in the fraction samples eluted from columns were calculated from the extinction values at 280 nm. All other protein determinations were by the method of Bradford (1976) with bovine serum albumin as calibration standard.

2.2c. Sodium determination

Na^+ concentrations in column fractions were measured by flame photometry using an IL343 photometer (Instrumentation Laboratory (UK) Ltd., Altrincham, Cheshire, U.K).

2.2d. Discontinuous SDS/polyacrylamide-gel electrophoresis

This was performed at room temperature (20°C) in the presence of 0.1% (w/v) sodium dodecyl sulphate (SDS)

1. See opposite page.

using vertical slab gels (0.075 cm x 16.5 cm x 18 cm) in an IN/96 electrophoresis apparatus (Raven Scientific Ltd., Haverhill, Suffolk, U.K.). The buffers used in the electrophoresis system were those described by Laemmli (1970). The 'resolving gel' was 14 cm long, of 12% (w/v) polyacrylamide and 2.6% (w/v) NN'-methylenebisacrylamide containing 375 mM Tris/HCl, pH 8.8. The 'stacking gel' was 1.5 cm high and comprised 3% (w/v) polyacrylamide containing 125 mM Tris/HCl, pH 6.8 and was formed on top of the 'resolving gel'.

Samples were prepared for electrophoresis by heating at 90°C for 5 min in an aqueous solution containing 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue and 10% (w/v) sucrose. Portions of these mixtures were run towards the anode through the 'stacking gel' (at 50 mA per gel) and then through the 'resolving gel' (at 25 mA per gel). Electrophoresis was stopped when the bromophenol blue dye marker had reached 0.5 cm from the end of the resolving gel. The gels were stained in a 0.2% (w/v) solution of Coomassie brilliant blue R in water/methanol/acetic acid (50:50:7, by vol) and destained in water/methanol/acetic acid (88:5:7, by vol.).

2.2e. Isoelectric focusing in polyacrylamide-gels

This was performed in 5% (w/v) polyacrylamide slab gels (12 cm x 11.5 cm x 0.2 cm) containing 10% (v/v) glycerol and 0.2% (w/v) Carrier Ampholines. Focusing was

carried out on an LKB 2117 Multiphor using an LKB 2103 power supply. Protein samples were subjected to isoelectric focusing in gels containing Ampholine mixtures which produced a broad pH range (pH 3.5 - 9.5). The electrode solutions were 1.0 M H_3PO_4 (anode solution) and 1.0 M NaOH (cathode solution). Samples (20 µl; approx. 25 µg of protein) were applied to strips (1 cm x 1 cm) of Whatman 3 MM paper placed 3 cm from the cathode electrode.

To ensure that protein focusing had been allowed to proceed to completion, some samples were also applied 3 cm from the anode electrode. The focusing was performed at 25 W for 3.5 h and the sample applicators removed after 1 h. The gels were fixed and stained according to the manufacturer's instructions (LKB Produktor application sheet 250).

2.2f. Analysis of amino acid compositions

The amino acid compositions of portions of homogenous GST isoenzymes were determined. The purified proteins were separated from substances of low molecular weight by extensive dialysis against 0.1 M ammonium acetate buffer pH 7.0. The samples for analysis were prepared simultaneously under identical conditions to permit direct comparison.

Portions (0.5 mg of protein) of GST were evaporated to dryness under vacuum in a desiccator, over H_2SO_4, at room temperature. These samples were hydrolysed at 105°C in evacuated sealed Pyrex glass tubes with 0.4 ml of 6 M HCl for 24 h as described by Ambler & Brown (1967). After

hydrolysis the glass tubes were opened and the samples were desiccated under vacuum, over NaOH pellets. The dried samples were then re-dissolved in 350 µl of 200 mM trisodium citrate/HCl buffer, pH 2.2, containing 0.5 mM L-amino guanidino-propionic acid and 0.5 mM L-norleucine as internal standards.

The amino acid compositions were determined using a Beckman-Spinco Model 120-C Amino Acid Analyser (Benson & Patterson, 1965). This instrument employs two ion-exchange columns: a 'short column' (0.9 cm x 5.0 cm which contained Pierce-Durram DA-X2 ion-exchange resin) separates the basic amino acids and ammonia and a 'long column' 0.9 cm x 50 cm, which contained Pierce-Durram DC-1A ion-exchange resin) separates the acidic and the neutral amino acids.

The complete amino acid compositons (basic amino acids plus acidic and neutral amino acids) of GST enzymes were obtained by dividing individual samples of hydrolysed protein into two 150 µl portions. One portion was eluted (70 ml/h, 53°C) from the short column with 116 mM trisodium citrate buffer, pH 5.2. The other portion was applied to the long column which was first eluted (70 ml/h, 53°C) with 67 mM trisodium citrate/HCl buffer, pH 3.28, until proline, glycine and alanine had eluted and was then eluted (70 ml/h, 53°C) with 67 mM trisodium citrate/HCl buffer, pH 4.25.

The recovery of the amino acids from each column was checked by comparing the elution of the two internal standards. The basic amino acid, L-amino guanidinopropionic acid, eluted from the short column between NH_3 and arginine. The acidic amino acid L-norleucine eluted from the long column between leucine and tyrosine. The recoveries were found to be 90 - 98%.

When pure peptides or proteins are hydrolysed certain amino acids are destroyed (Glazer et al., 1975). Asparagine is converted to aspartic acid and glutamine is converted to glutamic acid during hydrolysis. These two amino acids give rise to the ammonia released during the chemical hydrolysis of proteins. Since it was not possible to distinguish between the aspartic acid originally present in the basic GST and the aspartic acid produced as a result of acid hydrolysis of asparagine, the aspartic acid recovered from the long column during hydrolysis was designated Asx rather than Asp. Likewise, the glutamic acid recovered during analysis of GST was designated Glx rather than Glu.

Destruction of serine and threonine during hydrolysis occurs at a rate which is proportional to the temperature and time of heating. This loss can be allowed for by hydrolysing separate samples for different times and extrapolating recoveries to zero time of heating (Glazer et al., 1975). However, this was not possible during the present study due to paucity of material.

Cysteine and Cystine are acid labile and their loss during hydrolysis is difficult to quantitate. These amino acids are more reliably determined after first oxidising the samples of protein with performic acid (Hirs, 1967). This procedure converts both cysteine and cystine to cysteic acid and methionine to methionine sulphone. Cysteic acid, unlike cysteine and cystine, is not destroyed during hydrolysis in 6 M HCl and can be determined by measuring the cysteic acid content after performic acid oxidation. Tryptophan is rapidly destroyed during acid hydrolysis and has not been included in the analyses presented here.

Portions (2.5 mg of protein) of GST which had been freeze-dried were taken up in 250 µl of distilled water and treated with 250 µl 22 M performic acid (120 min, 0°c). Following oxidation, portions (100 µl, approx. 0.5 mg protein) were removed and desiccated for amino acid analysis. The remaining material was retained for peptide 'mapping'. The samples for amino acid analysis were then hydrolysed with 0.4 ml 6 M HCl (24 h, 105°C) before being desiccated and the dried products dissolved in 0.2 ml 200 mM trisodium citrate/HCl buffer, pH 2.2, containing 0.5 mM L-amino guanidinopropionic acid and 0.5 mM L-norleucine. A portion (0.15 ml; approx. 0.3 mg of protein) of the re-dissolved material was eluted from the long column (0.9 cm x 50 cm) of the Amino Acid Analyser.

Cysteic acid is the first amino acid to elute from the long column.

2.3 COLUMN CHROMATOGRAPHY AND ASSOCIATED TECHNIQUES

a. Anion-exchange chromatography

DEAE Sephadex A-50 was packed in columns (3.2 cm x 80 cm, or 2.2 cm x 30 cm) and equilibrated with 20 mM Tris/HCl pH 7.8. The larger column was eluted at 36 ml/h and the smaller column was eluted at 25 ml/h. These columns were developed by a 0 - 250 mM NaCl gradient, formed in the equilibration buffer, using 500 ml or 250 ml constant volume mixing reservoirs, for the large and small columns respectively. A small plug of Sephadex G25 was poured into each column and allowed to settle before the anion exchanger was poured; this prevented the DEAE-Sephadex beads from blocking the column support mesh.

DEAE-cellulose DE52 was packed in columns (4.4 cm x 75 cm) and equilibrated with 10 mM Tris/HCl buffer pH 8.0. Samples of protein were applied and the components which bound to the matrix were eluted (36 ml/h) by a 0 - 175 mM NaCl gradient formed in the equilibration buffer using a 500 ml constant volume mixing reservoir.

2.3b. Cation-exchange chromatography

CM-cellulose CM52 was packed in columns (2.2 cm x 24 cm) which had been equilibrated and eluted with 10 mM sodium phosphate buffer pH 6.7 at 25 ml/h. Protein was applied and the column developed by a 0 - 100 mM gradient

of NaCl formed in the equilibration buffer using a 250 ml constant volume mixing reservoir.

2.3c. Gel-filtration chromatography

Samples of protein were applied to columns (4.4 cm x 90 cm or 3.2 cm x 85 cm) of Sephadex G-100 equilibrated with 10 mM Tris/HCl pH 7.8. The proteins were eluted (48 ml/h) using the same buffer.

2.3d. S-Hexylglutathione affinity chromatography

Protein was applied to columns (1.6 cm x 20 cm) of S-hexylglutathione-Sepharose which had been equilibrated with 10 mM Tris/HCl buffer pH 7.8. The column was developed with two column volumes of running buffer before non-specifically adsorbed material was removed by eluting with two column volumes of running buffer containing 200 mM NaCl. Specifically bound protein was eluted with one column volume of running buffer containing 200 mM NaCl and 5 mM S-hexylglutathione.

S-hexylglutathione was first dissolved in a minimum volume of 1 M NaOH before adding it to the buffer. This solution was titrated to pH 7.8 using dropwise addition of HCl (11.8 M)

2.3e. Chromatofocusing

This was performed using either PBE 94 or PBE 118 anion exchange gel as directed by the manufacturer (Pharmacia Fine Chemicals AB). Table 2.2 summarises the conditions used for each type of anion exchanger. All

buffers and gels were de-gassed before use. The pH of the Start buffer and the pH of the Eluent buffer were chosen so that the proteins of interest would elute within the first half of the pH gradient.

The column matrix (either PBE 94 or PBE 118) was washed and equilibrated with the appropriate Start buffer before chromatography was commenced. A small volume (approx. 5 ml) of Eluent buffer was applied to the column before the protein sample, in order to commence formation of the pH gradient and avoid exposure of the protein to a high pH.

Table 2.2. Buffers and Gels for chromatofocusing in different pH ranges.

Gel	pH range	Start Buffer	Eluent Buffer
PBE 118	10.5-7.5	pH 11 25 mM triethyl/amine-HCl	pH 7.5 Pharmalyte pH 8-10.5/HCl
PBE 94	9-7	pH 9.4 25 mM ethanolamine/HCl	pH 7.0 Polybuffer 96/HCl
PBE 94	9-6	pH 9.4 25 mM ethanolamine/CH_3COOH	pH 6.0 Polybuffer 96/CH_3COOH
PBE 94	7-4	pH 7.4 25 mM imidazole/HCl	pH 4.0 Polybuffer 74/HCl

2.3f. Hydroxyapatite chromatography

Protein samples were applied to chromatography columns (2.2 cm x 20 cm) containing Bio-Rad HT grade hydroxyapatite which had been equilibrated with 10 mM sodium phosphate, buffer pH 6.7. The columns were developed with a 10 - 250 mM sodium phosphate pH 6.7 gradient using a 400 ml constant volume mixing reservoir.

2.3g. Ammonium sulphate fractionation

Solid $(NH_4)_2SO_4$ was added (4°C), with gentle stirring to the protein solution until 40% saturation was achieved (Dixon & Webb, 1979). This solution was left standing for 16 h (4°C) before the precipitate was removed by centrifugation (10 000 g, 15 min, 4°C) and discarded. More $(NH_4)_2SO_4$ was added to the resulting supernatant until 90% saturation was obtained. This solution was also left standing for 16 h (4°C) before centrifugation (10 000 g, 15 min, 4°C). The supernatant was discarded and the precipitate re-dissolved in 100-200 ml of 10 mM Tris/HCl buffer pH 7.8. The resulting solution is referred to as a "40% - 90% cut". In some preparations, for example the purification of GST B_1B_1 and B_1B_2, the protein solution was raised to 90% $(NH_4)_2SO_4$ saturation directly, without separating the material precipitated at 40% saturation.

2.3h. Concentration of protein samples by ultrafiltration

This was performed under nitrogen at 6 kPa using an Amicon ultrafiltration cell fitted with a PTGC membrane (molecular weight cut-off of Mr 10 000).

2.4 PURIFICATION OF HUMAN LIVER GST

a. Preparation of human liver cytosol and fractionation into DEAE-Pools 1, 2 and 3

Human liver (223 g) was chopped finely and homogenised in 500 ml of 10 mM Tris/HCl pH 8.0 at 4°C. The resulting solution was centrifuged at 10 000 g, for 30 min and the supernatant re-centrifuged at 100 000 g for 90 min. Lipid was removed from the 100 000 g supernatant by filtration through a plug of glass wool and the resulting solution was dialysed against two changes each, of 5 litres, of 10 mM Tris/HCl buffer pH 8.0 for 18 h.

The non-diffusible material (485 ml, about 10 g of protein) was applied to a column (4.4 cm x 75 cm) of DEAE-cellulose (flow rate 36 ml/h). Fractions of 9 ml were collected. The column was washed with 500 ml of running buffer before a 0-175 mM NaCl gradient was applied, using a 500 ml constant-volume mixing reservoir.

Three 'Pools' of transferase activity were eluted from DEAE-Cellulose and were defined as follows:-

DEAE-Pool 1: (fractions 80-155) transferases which did not bind to the anion exchanger and eluted in the flow-through fractions.

DEAE-Pool 2: (fractions 190-230) transferases which were slightly retarded by the column.

DEAE-Pool 3: (fractions 299-315) transferases which were bound by the column and eluted with the salt gradient between 50 mM - 70 mM NaCl.

Each of these pools of GST activity was separately subjected to several purification steps to yield homogenous glutathione S-transferases.

The following transferases were purified from each pool:-

GST B_1B_1 and B_1B_2 were obtained from DEAE-Pool 1.

GST B_2B_2 was obtained from DEAE-Pool 2.

GST N_2 was obtained from DEAE-Pool 3.

2.4b. Purification of basic GST B_1B_1 and B_1B_2

The enzyme fraction of human liver cytosol which did not bind to DEAE-Cellulose, Pool 1, was raised to 90% saturation with ammonium sulphate and the precipitate which was obtained was re-suspended in approx. 100 ml of 10 mM Tris/HCl buffer pH 7.8. This solution was dialysed against two changes, each of 2 litres, of the same buffer before being applied to a column (1.6 cm x 14 cm) of S-hexylglutathione-Sepharose affinity gel. The GST enzymes which were specifically eluted from the affinity matrix (approx. 16 mg of protein) were dialysed against two changes, each of 2 litres, of 10 mM Tris/HCl buffer pH 7.8 and applied to a column (2.2 cm x 30 cm) of DEAE-Sephadex.

The material which did not bind DEAE-Sephadex was eluted with 125 ml of running buffer. A gradient of 0 -175 mM NaCl was applied using a 500 ml mixing reservoir and eluted two major peaks of glutathione S-transferase activity between 25-45 mM NaCl and 50-60 mM NaCl; these

two peaks of activity were demonstrated to interconvert using CM-cellulose chromatography. Each peak was pooled separately and ultrafiltrated to a volume of approximately 10 ml before dialysis against two changes, each of 1 litre, of 10 mM sodium phosphate buffer pH 6.7 containing 2 mM GSH. The protein samples were then subjected to chromatography on columns (2.2 cm x 25 cm) of CM-cellulose which had been equilibrated with running buffer containing 2 mM GSH. A gradient of 0 - 100 mM NaCl formed in the running buffer, using a 500 ml constant volume mixing reservoir, was used to elute bound protein.

2.4c. Purification of GST B_2B_2 and N_2

The fraction of human liver cytosol which was retarded by DEAE-cellulose (DEAE-Pool 2) and the fraction which bound and was eluted on the salt gradient (DEAE-Pool 3), were separately subjected to essentially the same purification procedure. The basic-type GST, B_2B_2 was eluted in DEAE-Pool 2 whereas the neutral-type GST, N_2 was eluted in DEAE-Pool 3.

DEAE-Pool 2 and DEAE-Pool 3 were each subjected to ammonium sulphate precipitation. DEAE-Pool 2 was raised directly to 90% saturation and the precipitate obtained re-suspended in 20 ml of 10 mM Tris/HCl pH 7.8. DEAE-Pool 3 was subjected to a "40% - 90% cut" with ammonium sulphate and the precipitate was re-suspended in approx. 40 ml of the 10 mM Tris/HCl buffer.

Each sample of re-suspended protein was applied to a gel filtration column (3.2 cm x 85 cm) containing Sephadex G-100 and eluted as a single peak of glutathione S-transferase activity. The enzyme containing fractions from each column were separately pooled before being subjected to S-hexylglutathione affinity chromatography. The glutathione S-transferase containing fractions which were eluted with 5 mM S-hexylglutathione from each column were separately dialysed against two changes, each of 1 litre of 10 mM sodium phosphate buffer pH 6.7 before the final chromatographic step using columns (2.2 cm x 20 cm) of hydroxyapatite. In each case the glutathione S-transferase activity was bound by the matrix and was eluted by a gradient of 10 - 250 mM sodium phosphate buffer pH 6.7 using a constant volume 400 ml mixing reservoir.

2.4d. Purification of GST N_1

This neutral-type GST was purified from the N/A1 fraction of human liver cytosol by following the purification procedure, described in section 2.5b, to obtain glutathione S-transferase B_1B_2. Briefly, the method employed was DEAE-Sephadex chromatography, S-hexyl-glutathione affinity chromatography and chromatofocusing in the range pH 9-6.

2.5 REFERENCE METHODS FOR PURIFICATION OF HUMAN GST

Two purification methods have been reported in the literature (Kamisaka et al., 1975; Hayes et al., 1983) which have given rise to different nomenclatures for basic-type GST. The relationship between GST B_1B_1, B_1B_2, B_2B_2 and the human glutathione S-transferases which have previously been described was investigated.

2.5a. Preparation and identification of GST γ, δ and ϵ

Kamisaka et al. (1975) have used DEAE-Cellulose and CM-cellulose to resolve five GST forms (α, β, γ, δ and ϵ) from human liver. This purification scheme represents the basis of the most commonly used nomenclature to define the human glutathione S-transferases. It was used as described below to facilitate the identification of GST forms purified by other methods.

About 306 g of frozen liver was allowed to thaw at 20°C before being cut into small pieces and blended in 1.2 litres of ice-cold distilled water. All subsequent steps were carried out at 4°C. The extract was centrifuged at 10 000 g for 2 h and lipid was removed from the supernatant by filtration through a plug of glass wool. This preparation was applied to a column (3.2 cm x 85 cm) of DEAE-cellulose equilibrated with 10 mM Tris/HCl pH 8.0. The column was washed with the same buffer until free of transferase activity. These flow-through fractions from DEAE-cellulose were combined (821 ml) and con-

centrated by $(NH_4)_2$ SO_4 fractionation. The protein which precipitated at 95% $(NH_4)_2$ SO_4 saturation was dissolved in approximately 120 ml of 10 mM potassium phosphate pH 6.7 and dialysed for 20 h against three changes, each of 2 litres of the same buffer. The non-diffusible material (164 ml, about 0.3 g of protein) was applied to a column (3.2 cm x 56 cm) of CM-cellulose which had been equilibrated with 10 mM sodium phosphate pH 6.7. The column was washed with approximately 600 ml of the running buffer before a 0 - 150 mM KCl gradient was applied.

2.5b. Preparation of GST obtained from fractions N/A1, N/A2 and Basic

Hayes et al. (1983) purified six hepatic glutathione S-transferases from a single liver using DEAE-Sephadex (equilibrated at pH 7.8) chromatography as the principal resolution step. These enzyme forms were assigned to each of three groups; Basic, neutral/acidic 1 (N/A1) and neutral/acidic 2 (N/A2), based on their elution position from the anion exchanger. In this thesis, a purification strategy similar to that of Hayes et al. (1983) was used to further characterise these enzymes. However, no N/A2 peak was recovered from the liver examined.

About 200 g of human liver which had been stored frozen (-70°C) was allowed to thaw at 20°C before being cut into small pieces and blended in 400 ml of ice-cold 20 mM Tris/HCl buffer, pH 7.8. All subsequent steps were carried out at 4°C. The extract was centrifuged at

10 000 x g for 30 min and the supernatant was re-centrifuged at 100 000 x g for 90 min. Lipid was removed from the 100 000 x g supernatant by filtration through a plug of glass wool and the resulting solution was dialysed against two changes each of 5 litres of 10 mM Tris/HCl, pH 7.8 for 18 h.

The non-diffusible material (355 ml about 8 g of protein) was applied to a column (3.2 cm x 78 cm) of DEAE-Sephadex equilibrated and eluted 30.5 ml/h with 20 mM Tris /HCl buffer, pH 7.8. Transferase activity which did not bind to the column was eluted in the flow-through fractions from the column. The glutathione S-transferases that were retained by the DEAE-Sephadex column were eluted as a single peak of activity by a 0-250 mM NaCl gradient, formed using a 500 ml mixing reservoir. This peak which was eluted at an Na^+ concentration between 44 mM and 83 mM was identified as N/A1 according to the nomenclature used by the original authors (Hayes et al., 1983).

The flow-through fractions from the DEAE-Sephadex column were pooled and raised to 90% saturation with ammonium sulphate. The resulting precipitate was re-suspended in 100 ml of 20 mM Tris/HCl pH 7.8 and dialysed against three changes, each of 2 litres of the same buffer before being applied to an S-hexylglutathione affinity column. The glutathione S-transferase containing

fractions which were eluted with 5 mM S-hexylglutathione were combined and dialysed against two changes, each of 2 litres of 10 mM sodium phosphate buffer pH 6.7.

The N/A1 peak, which was eluted from the DEAE-Sephadex column at an Na^+ concentration between 44 mM and 83 mM, was pooled and applied to a S-hexylglutathione affinity column. The material eluted by 5 mM S-hexylglutathione was combined and dialysed against two changes, each of 1 litre of 5 mM Tris/HCl, pH 8.0, for 12 h. The non-diffusible material was concentrated to 5 - 10 ml by ultrafiltration and eluted from a chromatofocusing column (1.6 cm x 32 cm) by a pH 9.0 - 6.8 gradient.

2.6 REVERSIBLE DISSOCIATION AND SEPARATION OF GST

Analysis of GST B_1B_2

The subunits from GST B_1B_2 (approx. 0.5 mg of protein) were dissociated by incubation (1 h at 25°C) in a solution of 20 mM Tris/HCl pH 7.8, containing 6M-guanidinium chloride, 1 mM EDTA and 5 mM mercaptoethanol. The mixture was then dialysed against three changes each of 2 litres of 20 mM Tris/HCl pH 7.8 for 18 h to allow the subunits to re-associate. The products of this reversible dissociation were resolved by DEAE-Sephadex chromatography (Kitahara & Sato, 1981) using a column (2.2 cm x 21 cm) equilibrated with 20 mM Tris/HCl, pH 7.8 which was developed with a 0 - 250 mM gradient of NaCl using a 250 ml mixing reservoir.

[1]The blood was collected in plastic universal containers (25 ml) and allowed to clot at 4°C. Clot retraction was assisted by stirring with a glass rod before centrifugation at 3000 g to obtain the supernatant fraction (serum).

To facilitate the identification of each re-associated GST, portions (25-60 μg of protein) of these enzymes were dialysed against two changes each of 1 litre of 10 mM sodium phosphate, pH 6.7 before being applied to identical columns (2.2 cm x 24 cm) of CM-cellulose equilibrated with the same buffer. A 0 - 100 mM NaCl gradient, formed using a 250 ml mixing reservoir, was used to develop these columns.

2.7 IMMUNOCHEMICAL TECHNIQUES

a. Preparation of antisera

Antisera employed in this study against GST B_1B_1 and B_2B_2 were those described by Hayes et al. (1983) and Beckett & Hayes (1984). During this thesis antisera were raised against GST N_1 and N_2 as follows. Portions (0.2 mg/ml) of purified GST were separately emulsified with an equal volume of Freund's complete adjuvant. Approximately 200 μg of each of the emulsions was injected sub-cutaneously at eight separate sites on the back of female New Zealand white rabbits. After 6 weeks each rabbit was re-inoculated with 200 μg of the original immunogen in incomplete Freund's adjuvant, and 2 weeks later the animals were killed and bled.[1] The serum which was obtained was stored at -70°C.

2.7b. 'Western' blotting

The immunological relationship between the purified hepatic transferases was examined by using the 'Western'

blotting technique, first described by Towbin et al. (1979). The transferase subunits were resolved by discontinuous SDS/polyacrylamide-gel electrophoresis. Following electrophoresis, the polyacrylamide-gel slabs were equilibrated with a solution containing 25 mM Tris/193 mM-glycine, pH 8.3/20% (v/v) methanol, for 30 min at room temperature. The polypeptides were then electrophoretically transferred to nitrocellulose, in the same equilibrium buffer, by using a Bio-Rad model 250/2.5 power supply (Bio-Rad Laboratories). The Trans-Blot Cell was assembled according to the manufacturer's instructions. However, in certain experiments, 2 nitrocellulose sheets were placed on top of the gel; normally a single sheet is used. Quantitative transfer of protein from the polyacrylamide-gel onto several sheets of nitrocellulose has been achieved by other workers (T J Mantle, Personal Communication), thereby permitting several copies of a single electrophoretic transfer to be made simultaneously.

Transfer of protein to nitrocellulose paper was performed for 4 h at 15°C with 0.25A. Free binding sites on the nitrocellulose paper were blocked (16 h, 20°C) by using a 3% (w/v) solution of gelatin in buffer A (20 mM Tris/HCl buffer, pH 7.5, containing 500 mM-NaCl and 0.05% Tween-20).

After blocking with gelatin, each nitrocellulose paper was incubated (2.5 h, 20°C) with primary antibody raised

against either GST B_1B_1, B_2B_2, N_1 or N_2. The antisera were diluted in buffer A, containing 1% (w/v) gelatin as given in Table 2.3. The polypeptides that cross-reacted with these antisera were detected with the Bio-Rad goat anti-(rabbit IgG) antibody-horseradish-peroxidase conjugate immunoblot assay kit.

Table 2.3. Dilution of primary antibody for 'Western' blot analysis

Primary antibody	Dilution
anti-B_1	1:3000
anti-B_2	1:1500
anti-N_1	1: 750
anti-N_2	1: 750

2.7c. Radioimmunoassay of GST isoenzymes

Radioimmunoassay of various glutathione S-transferases was carried out as described previously by Hayes et al. (1983) by using antisera raised to transferase B_1B_1 and to B_2B_2 [previously referred to as Basic and N/A2b respectively (Hayes et al., 1983)].

All assay constituents were made up in 50 mM potassium phosphate, pH 7.5, containing bovine serum albumin (1 g/litre) and sodium azide (0.2 g/litre). Sample or standard (100 µl) was incubated with antisera (100 µl of a 1:5000 dilution, containing normal rabbit serum at a 1:300 dilution) for 48 h at 4°C. Tracer (50 µl; 10 000 dpm) was then added and the incubation was continued for a

further 48 h at 4°C. Donkey anti-rabbit antiserum (100 µl of a 1:30 dilution) was then added and after a further 24 h at 4°C a wash solution (2 ml; 0.05% aqueous Brij 35 solution) was added and the tubes centrifuged for 30 min (4°C, 3000 x g). The supernatant was decanted and the precipitate counted in a Nuclear Enterprises 1600 multidetector gamma counter.

The relative cross-reactivity was assessed by calculating the amount of protein that was required to produce 50% displacement of bound ligand. The percentage cross-reactivity was determined by comparing this value with that obtained from the most immunoreactive protein.

2.8 PEPTIDE 'MAPPING' OF GST ENZYMES

Portions (approx. 2.0 mg) of GST B_1B_1, B_1B_2 and B_2B_2 were dried under vacuum in a desiccator over H_2SO_4. The three proteins were prepared for proteolysis by oxidation with performic acid (120 min, 0°C) and the reaction products desiccated under vacuum. The proteins were dissolved in 100 µl 0.1 M NH_3 before 0.3 ml of 200 mM ammonium acetate buffer, pH 8.5, was added and the pH adjusted to 9 - 10. with 0.1 M NH_3.

Digestion of B_1B_1, B_1B_2 and B_2B_2 was carried out at 37°C for 18 h with trypsin using a protein/proteolytic enzyme ratio of 40:1 (w/w)(Bhargava et al., 1978, Hayes et al., 1981). After digestion, the reaction

mixture was lyophilised and stored under vacuum at room temperature.

The freeze-dried digestion products were examined by high-voltage paper electrophoresis after dissolution of the freeze-dried powder in 100 µl 0.1 M NH_3. This solution was centrifuged (4000 g, 2 min, 20°C) and the supernatant applied to Whatman 3 MM paper (56 cm long, 20-50 cm wide). The samples were subjected to high-voltage paper electrophoresis which was carried out in apparatus similar to that described by Allen (1981). The buffer systems and coolants at pH 6.5 and 3.5 were as described by Ambler (1963). In all the runs (45 min at 3000V), a parallel separation of a mixture of amino acids and dyes including arginine, histidine, ε-DNP-lysine, aspartic acid, glutamic acid and xylene cyanol FF ('Wondermix') and a spot of red Pentel pen was performed on the same paper (Milstein & Milstein, 1968). The relative mobilities of these markers is given in Figure 2.1. After each run the paper was examined under ultraviolet light (340 nm) before ninhydrin treatment. Tryptophan-containing peptides fluoresce at this wavelength (Ambler, 1963). Peptides were stained by dipping the paper in 0.2% (w/v) ninhydrin solution in acetone to which had been added (just before use) 3% (v/v) collidine. The paper was then dried at room temperature, for 5 min, and then heated at 70°C for 10 min.

Figure 2.1. Relative mobilities of reference markers used for high-voltage paper electrophoresis.

Electrophoresis of 'Wondermix' was carried out at either pH 6.5 or pH 3.5 using the buffer systems and coolants described by Ambler (1963).

Single-letter abbreviations for the amino acid markers are used in the figures of the peptide 'maps'.

Additional abbreviations for dyes ε-DNP-lysine (ε); red Pentel marker, (P); xylene cyanol FF, (X).

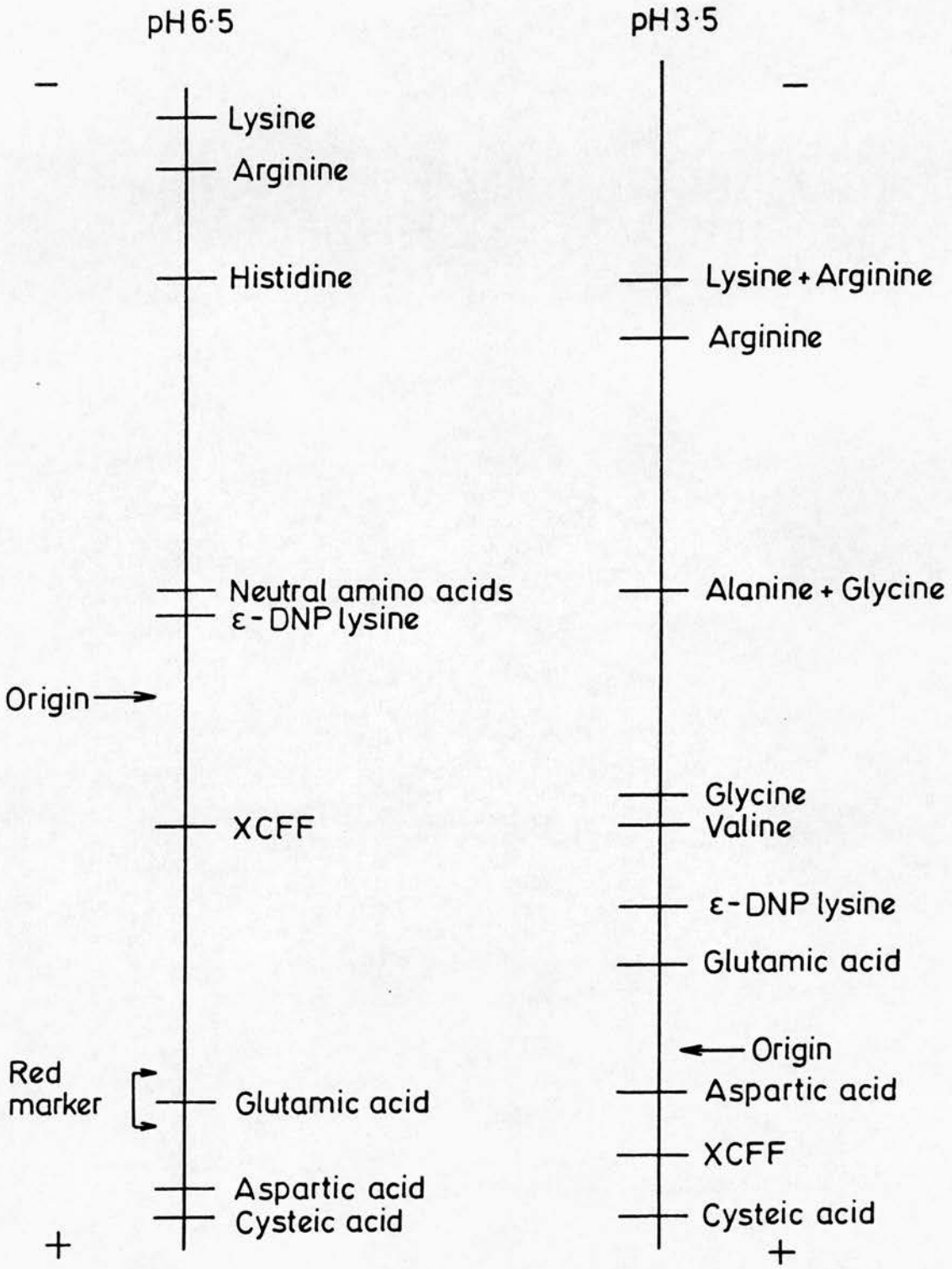

pH 6·5
−
Lysine
Arginine
Histidine
Neutral amino acids
ε-DNP lysine
Origin
XCFF
Red marker
Glutamic acid
Aspartic acid
Cysteic acid
+
pH 3·5
−
Lysine + Arginine
Arginine
Alanine + Glycine
Glycine
Valine
ε-DNP lysine
Glutamic acid
Origin
Aspartic acid
XCFF
Cysteic acid
+

Figure 2.1

Multi-dimensional tryptic peptide 'maps' of GST B_1B_1, B_1B_2 and B_2B_2 were formed by a combination of electrophoresis and chromatography. All steps involved in the 'mapping' were performed simultaneously on three GST forms under identical conditions. A diagrammatic representation of the procedures involved in constructing these 'maps' is shown in Fig. 2.2. In the first dimension the soluble peptides were separated into the basic, neutral and acidic tryptic peptides by high-voltage paper electrophoresis at pH 6.5. During electrophoresis at pH 6.5, the basic peptides migrate towards the cathode, the acidic peptides migrate towards the anode and the neutral peptides migrate as a single band which is slightly displaced from the origin (towards the cathode) by endosmosis.

The acidic peptides from the different GST forms, were partially resolved by electrophoresis at pH 6.5. These were separated in a second dimension, at right angles to the first, by high-voltage paper electrophoresis at pH 3.5. This procedure was carried out by cutting the series of acidic peptides from the electrophoresis paper after the first dimension. The peptides were then divided into two portions, ATP1 and ATP2, to enable the three proteins to be compared on a single sheet of paper. All the ATP1 portions were rotated through 90° from the first dimension and sewn onto fresh paper in a head-to-

Figure 2.2. Scheme for the construction of the multi-dimensional peptide 'maps' of GST B_1B_1, B_1B_2 and B_2B_2. For the sake of simplicity the diagram only shows the construction of peptide 'map' for a single protein. However, in practice all three proteins were subjected to the same procedure in parallel.

The tryptic digests of B_1B_1, B_1B_2 and B_2B_2 were separated in the first dimension (1) by high-voltage paper electrophoresis at pH 6.5 into basic tryptic peptides (BTP), neutral tryptic peptides (NTP) and acidic tryptic peptides (ATP). The BTP were then subjected to descending chromatography (2) using butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.). The NTP were subjected to electrophoresis at pH 3.5 (2) followed by descending chromatography (3). The ATP were subjected to electrophoresis at pH 3.5 (2). The diagram is not drawn to scale.

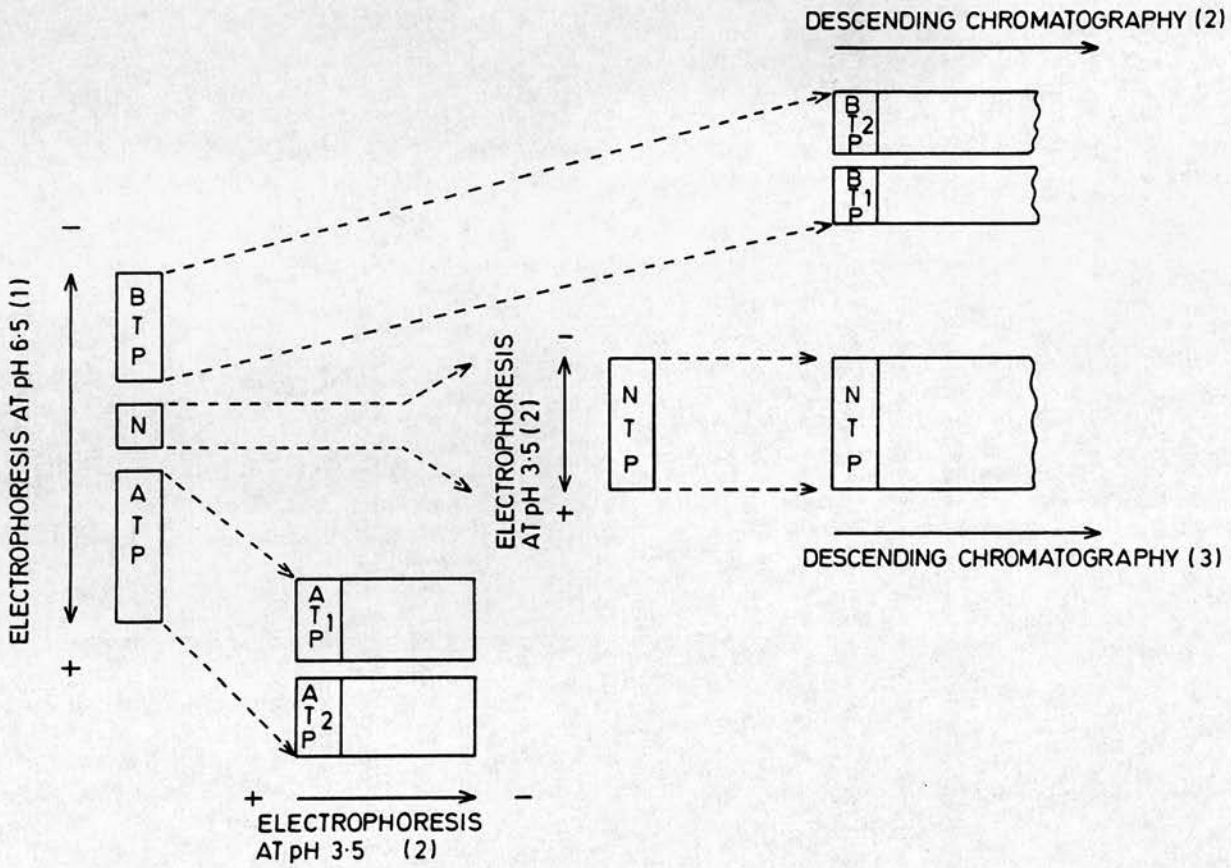

DESCENDING CHROMATOGRAPHY (2)
BTP2
BTP1
ELECTROPHORESIS AT pH 6·5 (1)
BTP
N
ATP
ELECTROPHORESIS AT pH 3·5 (2)
NTP
NTP
DESCENDING CHROMATOGRAPHY (3)
ATP1
ATP2
ELECTROPHORESIS AT pH 3·5 (2)

Figure 2.2

tail orientation. The same procedure was followed for the ATP2 portions. All the acidic peptides were then subjected to electrophoresis in the second dimension.

The basic peptides from the different GST forms were also partially resolved by electrophoresis at pH 6.5. These were separated in a second dimension by descending chromatography. Following the first dimension, the basic peptides were divided into two portions BTP1 and BTP2. All the BTP1 portions were sewn onto a single piece of chromatography paper in a head-to-tail orientation and the same procedure was followed for the BTP2 portions. All the basic peptides were then subjected to descending chromatography using butan-1-ol/acetic acid/water/pyridine (15:3:12:10 by vol.) for 16 h in the second dimension.

Since individual neutral tryptic peptides (NTP) were not resolved by electrophoresis at pH 6.5, they were sewn onto chromatography paper and separated by high-voltage paper electrophoresis at pH 3.5 in the same orientation as the first dimension. The neutral peptides were then further separated by descending chromatography using butan-1-ol/acetic acid/water/pyridine (15:3:12:10 by vol.) for 16 h in a third dimension at right angles to the direction of electrophoresis.

SECTION 3: RESULTS

3.1 PURIFICATION OF GST B_1B_1, B_1B_2, B_2B_2, N_1 and N_2

A summary of the chromatographic properties of the GST forms purified during this thesis is presented in section 3.1c and Table 3.1.

3.1a. Purification procedure of basic GST enzymes

DEAE-cellulose chromatography of human liver cytosol at pH 8.0 resolved three pools of glutathione S-transferase activity (Fig. 3.1). These peaks were designated DEAE-cellulose Pools 1, 2 and 3 by their elution position from this anion exchanger; DEAE-cellulose Pool 1 did not bind to the column and was eluted in the flow-through fractions. DEAE-cellulose Pool 2 was slightly retarded by the column. DEAE-cellulose Pool 3 was bound by the column and was eluted by the salt gradient. GST B_1B_1 and B_1B_2 were purified from DEAE-cellulose Pool 1, transferase B_2B_2 was purified from Pool 2 and transferase N_2 was purified from Pool 3.

Transferases B_1B_1 and B_1B_2, recovered from the DEAE-cellulose Pool 1, were purified by ammonium sulphate fractionation, S-hexylglutathione affinity chromatography and DEAE-Sephadex chromatography. The 0-90% ammonium sulphate "cut" of DEAE-cellulose Pool 1 was eluted from S-hexylglutathione affinity gel with 80% recovery of GST activity. DEAE-Sephadex chromatography resolved three peaks of activity; the first, which comprised transferase B_1B_1, did not bind to the column and eluted in the flow-through

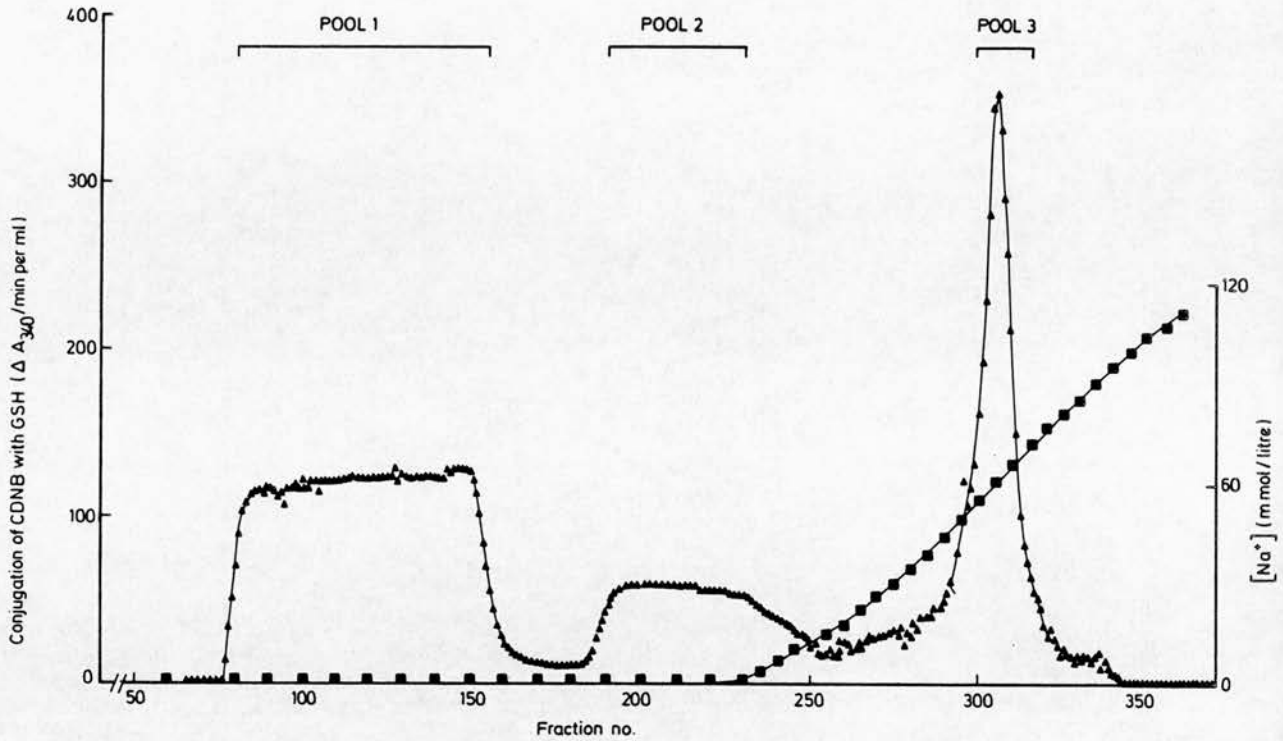


Figure 3.1. Elution profile of cytosolic GST in human liver from DEAE-cellulose.

Hepatic cytosol was dialysed against 10 m<u>M</u> Tris/HCl buffer pH 8.0 and the resulting material (about 500 ml) was applied to a 4.4 cm x 75 cm column of DEAE-cellulose. The column was washed with 500 ml of running buffer before a 0-175 m<u>M</u> NaCl gradient was applied. Fractions of 9.0 ml were collected. Three pools of GST activity with 1-chloro-2,4-dinitrobenzene (CDNB, ▲) were resolved. GST B_1B_1 and B_1B_2 were obtained from DEAE-Pool 1. GST B_2B_2 was obtained from DEAE-Pool 2 and GST N_2 was obtained from DEAE-Pool 3. The Na^+ concentration [■] was also measured.

fractions. The second and third peaks were eluted by the salt gradient at sodium concentrations of 25-45 mM and 50-60 mM respectively (Fig. 3.2). The latter two peaks of activity were both found to comprise transferase B_1B_2. Interconversion of these peaks occurred when they were separately re-subjected to anion exchange chromatography suggesting that they did not possess different primary structures.

Glutathione S-transferases B_2B_2 and N_2 were purified from DEAE-cellulose Pool 2 and Pool 3 respectively by ammonium sulphate fractionation, gel filtration on Sephadex G-100 chromatography, S-hexylglutathione affinity chromatography and hydroxyapatite chromatography. In each case, the pool of activity obtained following the ammonium sulphate cut was found to elute as a single symmetrical peak from the gel filtration column. Purification using S-hexylglutathione affinity chromatography resulted in approximately 80% yield of GST activity. On the final purification step, using hydroxyapatite chromatography, the material from Pool 2 (GST B_2B_2) eluted as a single peak of activity at a sodium concentration of 254 mM (Fig. 3.3b). By contrast, Pool 3 (GST N_2) eluted from the hydroxyapatite column as a major peak of activity at a sodium concentration of 139 mM. Pool 3 also yielded a minor peak which eluted at a position identical to the position identical to GST B_2B_2 (Fig. 3.3a); this was thought to represent contamination from Pool 2.

Figure 3.2. Elution profile of DEAE-Pool 1 from DEAE-Sephadex.

Human liver GST activity which did not bind to DEAE-cellulose at pH 8.0 was subjected to ammonium sulphate precipitation (0-90% "cut") followed by S-hexylglutathione affinity chromatography. The material which was specifically eluted from the affinity matrix (approx. 14 mg of protein) was applied to a column (2.2 cm x 30 cm) of DEAE-Sephadex. The material which did not bind to this matrix was eluted with 125 ml of 10 mM Tris/HCl pH 7.8. A gradient of 0-175 mM NaCl was applied using a 500 ml mixing reservoir. Fractions (5.5 ml) were collected and the glutathione S-transferase activity with 1-chloro-2,4-dinitrobenzene (▲) and the Na^+ concentration (■) were measured.

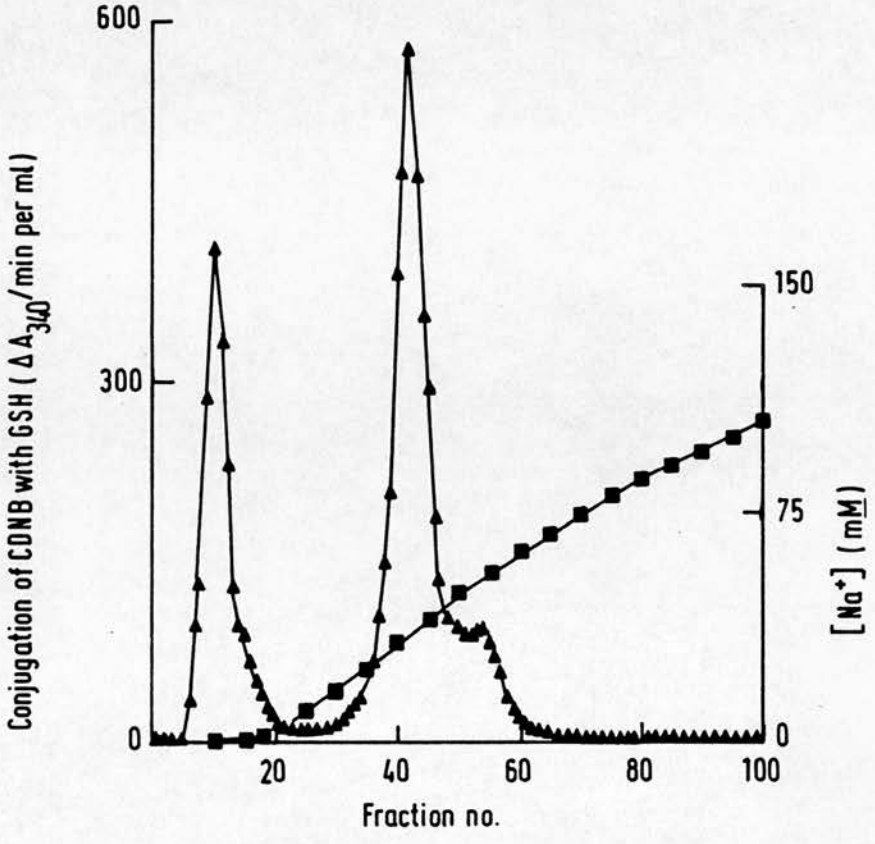

600
300
0
Conjugation of CDNB with GSH (ΔA_{340}/min per ml)
150
75
0
[Na⁺] (mM)
20
40
60
80
100
Fraction no.

Figure 3.2

Figure 3.3. Elution profile of DEAE-Pool 2 and DEAE-Pool 3 from hydroxyapatite.

The fraction of human liver cytosol which was retained by DEAE-cellulose (DEAE-Pool 2) and the fraction which bound and was eluted on the salt-gradient (DEAE-Pool 3) were separately subjected to ammonium sulphate fractionation, gel filtration on Sephadex G-100 and S-hexylglutathione affinity chromatography. The GST containing fractions which were eluted by 5 m<u>M</u> S-hexylglutathione, were separately dialysed against 2 x 1 litre of 10 m<u>M</u> sodium phosphate pH 6.7. DEAE-Pool 2 (b) (approx. 5 mg of protein) and DEAE-Pool 3 (a) (approx. 4 mg of protein) were applied to columns (2.2 cm x 20 cm) of hydroxyapatite and eluted by a gradient of 10-250 m<u>M</u> sodium phosphate pH 6.7. Fractions (5.3 ml) were collected and the [Na^+] (▲) and transferase activity with CDNB (■) were determined.

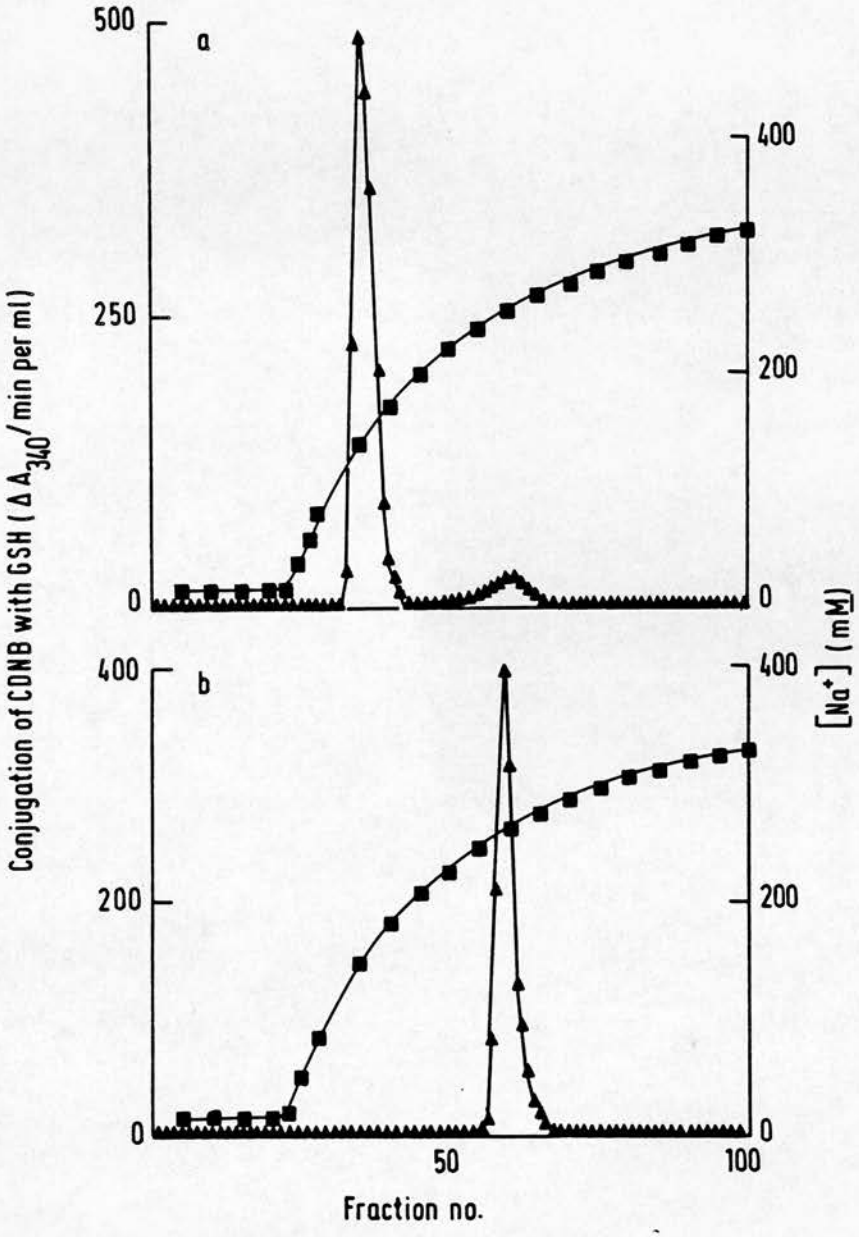

a
b
500
250
0
400
200
0
400
200
0
400
200
0
50
100
Fraction no.
Conjugation of CDNB with GSH (ΔA_{340}/min per ml)
[Na^+] (mM)

Figure 3.3

3.1b. Purification of neutral GST N_1

Glutathione S-transferase N_1 was obtained from the N/A1 fraction of human liver cytosol eluted from DEAE-Sephadex chromatography (Fig. 3.4). The N/A1 fraction which eluted from DEAE-Sephadex at a Na^+ concentration between 44 mM and 88 mM was further purified by S-hexyl-glutathione affinity chromatography and chromatofocusing over the pH range 9.0 - 6.8. Glutathione S-transferase N_1 was resolved from GST B_1B_2 during the final chromato-focusing step; GST N_1 eluted at pH 7.7 (Fig. 3.5) and GST B_1B_2 at pH 8.3 (Fig. 3.6). GST B_1B_1 was obtained from the flow-through fractions (basic pool) from DEAE-Sephadex. Further purification of GST B_1B_1 involved ammonium sulphate fractionation and S-hexylglutathione chromatography.

3.1c. Chromatographic properties of hepatic GST

Detailed information about the elution positions of glutathione S-transferases from various types of chroma-tography column is a useful method for identification of these enzymes.

The purification of each human liver glutathione S-transferase required several types of chromatographic separation. Table 3.1 summarises the chromatographic behaviour of each hepatic GST. These results were obtained from the purification of the enzymes and by sub-jecting portions of purified enzymes to column matrices

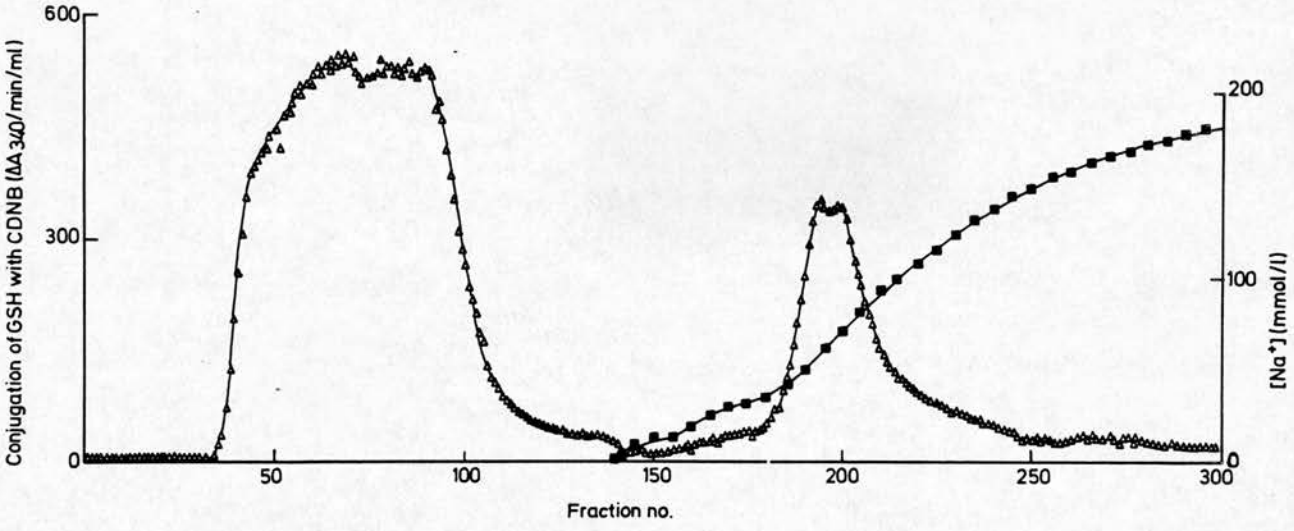


Figure 3.4. Elution profile of cytosolic GST in human liver from DEAE-Sephadex.

Human liver cytosol (approx. 8 g of protein) was dialysed against 20 mM Tris/HCl pH 7.8 and eluted from a column (3.2 cm x 78 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. The column was developed with a 0 - 250 mM NaCl gradient. Fractions (5.4 ml) were collected. The Na^+ concentration (■) and GST activity with CDNB (▲) were determined.

Flow-through material corresponds with the Basic pool and the gradient peak (fractions, 185-210) corresponds to the N/A1 pool, previously described by Hayes et al. (1983). This elution profile was obtained from a liver which did not express GST in the N/A2 pool.

Figure 3.5. Chromatofocusing of glutathione S-transferase N_1.

The glutathione S-transferases that were retained by DEAE-Sephadex and eluted on the salt gradient (at [Na+] 44-83 mM) were purified by S-hexylglutathione affinity chromatography. The resulting material (about 10 mg of protein) was dialysed against 2 x 1 litre of 25 mM ethanolamine/acetate, pH 9.0, and applied to a column (1.6 cm x 45 cm) of chromatofocusing gel PBE 94. This column was eluted (18 ml/h) with Polybuffer 96 adjusted with acetic acid to pH 6.5, and 5.8 ml fractions were collected. The pH (●) and transferase activity with CDNB (▲) in the eluate were determined. This elution profile was obtained from a liver whose phenotype was different from that of the liver used for Fig. 3.6; it contained little transferase B_1B_2.

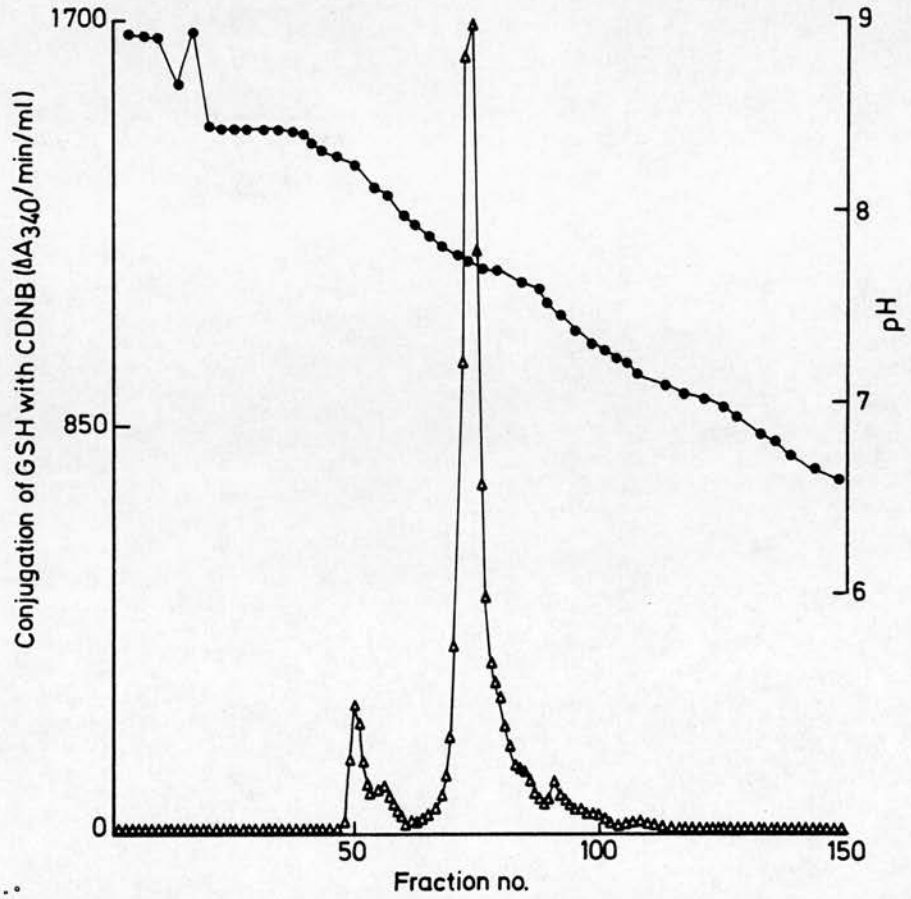

1700
850
0
9
8
7
6
pH
Conjugation of GSH with CDNB (ΔA340/min/ml)
50
100
150
Fraction no.

Figure 3.5

Figure 3.6. Chromatofocusing of glutathione S-transferase B_1B_2.

The glutathione S-transferases that were retained by DEAE-Sephadex and eluted on the salt gradient (at [Na+] 44-83 mM) were purified by S-hexylglutathione affinity chromatography. The resulting material (about 7 mg of protein) was dialysed against 2 x 1 litre of 5 mM Tris/HCl, pH 8.0, and, after concentration to about 5 ml, was applied to a column (1.6 cm x 32 cm) of chromatofocusing gel PBE 94. This column was equilibrated with 25 mM ethanolamine/acetate, pH 9.4, and eluted (27 ml/h) with Polybuffer 96 adjusted with acetic acid to pH 6.8. Fractions of volume 6.6 ml were collected. The pH (●) and transferase activity with CDNB (△) were determined. This elution profile was obtained from a liver whose phenotype was different from that of the liver used for Fig. 3.5; it did not express transferase N_1.

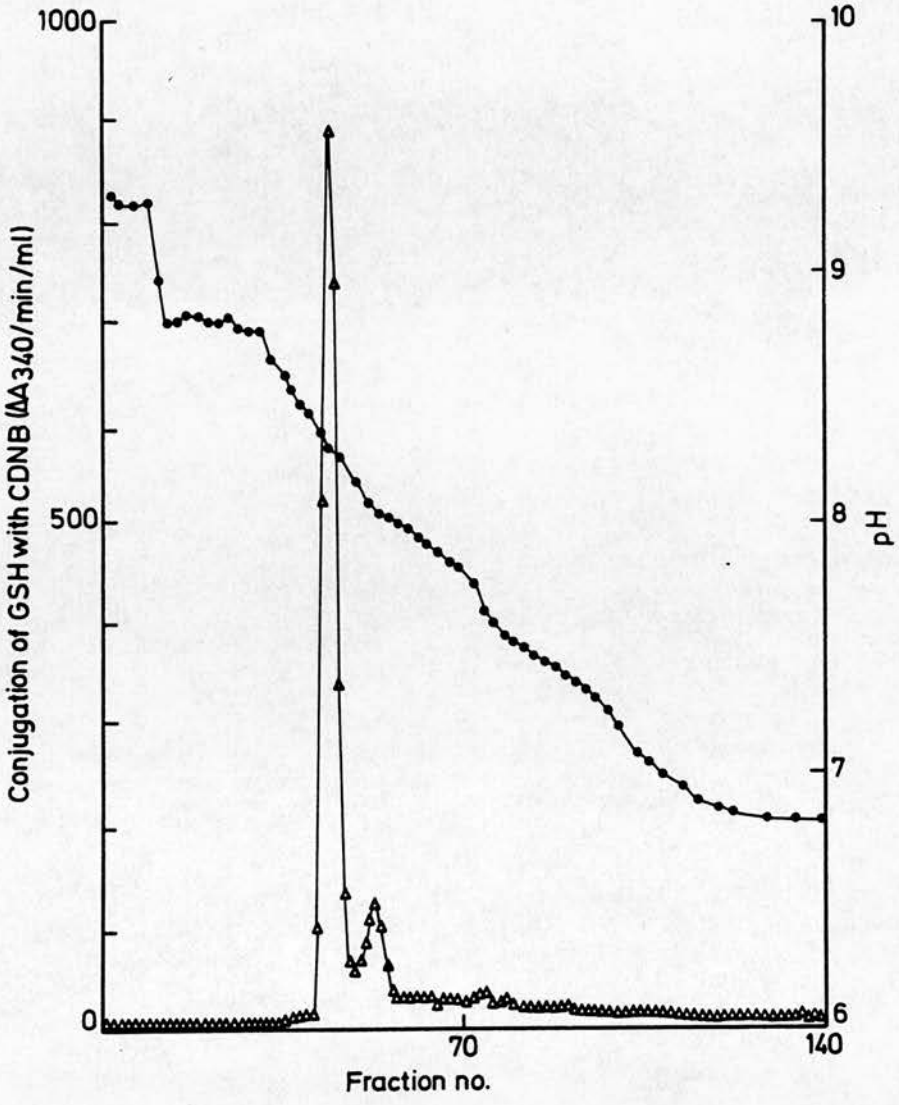

1000
500
0
10
9
8
7
6
pH
70
140
Fraction no.
Conjugation of GSH with CDNB (ΔA340/min/ml)

Figure 3.6

Type of Chromatography	Transferase				
	B_1B_1	B_1B_2	B_2B_2	N_1	N_2
DEAE-Sephadex pH 7.8	Flow-through	Interconversion; 2 peaks $[Na^+]$20-85 mM	$[Na^+]$120-150 mM	$[Na^+]$20-50 mM	$[Na^+]$20-90 mM
DEAE-cellulose pH 8.0	Flow-through	Flow-through	$[Na^+]$0-15 mM	$[Na^+]$50-70 mM	$[Na^+]$50-70 mM
CM-cellulose pH 6.7	$[Na^+]$44-48 mM	Interconversion; 2 peaks $[Na^+]$20-40 mM	Flow-through	N.D.	N.D.
Hydroxyapatite pH 6.7	$[Na^+]$245-270 mM	$[Na^+]$245-270 mM	$[Na^+]$245-270 mM	$[Na^+]$110-150 mM	$[Na^+]$110-150 mM
Chromatofocusing	pH 9.0	pH 8.3	pH 5.3	pH 7.7	pH 6.8*
S-hexylglutathione affinity	Binds	Binds	Binds	Binds	Binds

Table 3.1. Elution position of glutathione S-transferases.

Summary of the chromatographic properties of human liver glutathione S-transferases. The details of the procedures used with each type of column chromatography are given in the Materials Methods section. Interconversion is discussed in section 3.1d. Column dimensions used were as follows; DEAE Sephadex, 3.2 cm x 80 cm; DEAE-cellulose, 4.4 cm x 75 cm; CM-cellulose, 2.2 cm x 24 cm; Hydroxyapatite, 2.2 cm x 20 cm; Chromatofocusing, 1.6 cm x 32 cm; S-hexylglutathione affinity, 1.6 cm x 20 cm. N.D. denotes values which were not determined.

*The elution position of transferase N_2 was obtained by Dr. J.D. Hayes.

which were not used in their preparation. All five forms of human transferase bind the affinity matrix S-hexyl-glutathione-Sepharose and this matrix provides a simple, rapid method for obtaining GST from tissue cytosol.

Hydroxyapatite chromatography was found to resolve the hepatic transferases into two groups. The first group of GST co-elute at sodium concentration of 245-270 m$\underline{M}$ (transferases B_1B_1, B_1B_2 and B_2B_2), and the second group of GST co-elute at a sodium concentration of 110-150 m$\underline{M}$ (transferases N_1 and N_2).

The anion exchange matrices DEAE-Sephadex and DEAE-cellulose were found to differ in their affinity for the enzymes. Since DEAE-Sephadex was equilibrated with Tris/HCl buffer pH 7.8 and DEAE-cellulose was equilibrated with Tris/HCl buffer pH 8.0, the variations in chromatographic behaviour probably reflect hydrophobic rather than ionic differences between the GST. Transferase B_1B_1 was not retained by either DEAE-Sephadex or DEAE-cellulose. By contrast, GST B_1B_2 was retained by DEAE-Sephadex but was not bound by DEAE-cellulose under the conditions employed. GST B_1B_1 was the most basic form of enzyme obtained from chromatofocusing and other types of column as judged by elution position. GST B_1B_2 appeared to be less basic than B_1B_1 and was also found to separate into several peaks of activity from the ion-exchange resins to which it bound. The chroma-

tographic properties of transferase B_2B_2 were peculiar, making it difficult to interpret column profiles of mixtures of GST. For example, GST B_2B_2 behaved as a more acidic protein than GST N_1 when analysed using chromatofocusing or DEAE-Sephadex chromatography, but this relationship was reversed when GST B_2B_2 and N_1 were analysed using DEAE-cellulose.

Transferases N_1 and N_2 were found to elute in different positions from the chromatofocusing column, at pH values close to neutrality. These two enzymes were found to co-elute from all other column matrices studied and therefore chromatofocusing was found to be the method of choice to resolve GST N_1 and N_2.

3.1d. Interconversion of the multiple forms of GST B_1B_1 and B_1B_2

Transferases B_1B_1 and B_1B_2 were often found to give rise to multiple peaks of activity when separately subjected to ion-exchange chromatography. This phenomenon was most apparent with samples of enzyme which had been stored at 4°C for several days. However, it was found that if the enzymes were incubated and re-subjected to chromatography in the presence of 2 mM glutathione these multiple peaks of activity converged to yield a single form. When this experiment was repeated using 2 mM dithiothreitol instead of glutathione, no alteration in the multiple enzyme profile occurred.

3.2 FUNCTIONAL ANALYSIS OF HUMAN GST

a. Substrate specificities

The specific activity of each purified form of human liver glutathione S-transferase was determined with a range of substrates. The results were analysed to identify if functional differences exist between the different enzyme forms and between the basic enzyme subunits B_1 and B_2.

Table 3.2 lists the specific activity of each human liver enzyme for a particular substrate, also included for comparison are the values obtained for the acidic transferase from human lung GST (λ). All of the transferases were highly active with 1-chloro-2,4,-dinitrobenzene however some enzyme forms exhibited little or no detectable activity with other substrates. A comparison of the catalytic properties of these enzymes indicated a close functional homology between transferases B_1B_1, B_1B_2 and B_2B_2 and also between transferases N_1 and N_2. Transferases B_1B_1, B_1B_2 and B_2B_2 were characterised by a high glutathione peroxidase activity with cumene hydroperoxide as substrate and no detectable activity with trans-4-phenyl-3-buten-2-one or 1,2-epoxy-3-(p-nitrophenoxy)-propane. The basic enzymes were also less active with 1-chloro-2,4,dinitrobenzene and more active with 1,2-dichloro-4-nitrobenzene than the other enzymes studied. By contrast, transferases N_1 and N_2 were characterised by a high specific activity with trans-4-phenyl-3-buten-2-one and were inactive with 1,2-dichlor-

Substrate	Specific activity (μmol.min^{-1}.mg^{-1}) at 37°C					
	Transferase					
	B_1B_1	B_1B_2	B_2B_2	N_1	N_2	λ
1-Chloro-2,4-dinitrobenzene	82	117	80	272	143	212
1,2-Dichloro-4-nitrobenzene	0.25	0.86	0.79	0	0	0.14
Ethacrynic acid	0.11	0.16	0.14	0.22	0.024	1.22
trans-4-Phenyl-3-buten-2-one	0	0	0	0.45	0.25	0.021
1,2-Epoxy-3-(p-nitrophenoxy)-propane	0	0	0	N.D.	0.29	0.53
Cumene hydroperoxide	30.7	91.6	103.7	N.D.	0.34	0.11
p-Nitrophenyl acetate	0.66	0.97	0.24	N.D.	0.058	0.38

Table 3.2. Substrate specificities of human glutathione S-transferases. Transferase activity was determined as described in section 2.2a. N.D. denotes values which were not determined due to shortage of material.

4-nitrobenzene. None of these enzymes displayed a high specific activity with ethacrynic acid, the property which is characteristic of transferase λ.

Although the B_1 and B_2 subunits were active with the same spectrum of substrates, differences in their specific activities were apparent with certain substrates which permitted them to be discriminated. The B_2 subunit is approximately 3-fold more active with 1,2-dichloro-4-nitrobenzene and cumene hydroperoxide and approximately 2.5-fold less active with p-nitrophenyl acetate than the B_1 subunit.

The specific activity of the B_1B_2 hybrid enzyme in each case was greater than the calculated sum of the activities of the separate B_1 and B_2 subunits and suggested that a functional co-operativity may occur between these subunits.

A full comparison between transferases N_1 and N_2 was not performed because of paucity of material. However, it was found that transferase N_1 is approximately ten times more active than transferase N_2 with ethacrynic acid.

3.2b <u>The effects of specific inhibitors of GST activity</u>

A series of compounds which are known to selectively inhibit the activity of individual rat GST subunits was tested on transferases B_1B_1, B_1B_2 and B_2B_2. The aim of this experiment was to detect if the B_1 and B_2 subunits were equally sensitive to these inhibitors.

Table 3.3 gives the results for each of the basic human GST B_1B_1, B_1B_2 and B_2B_2 assayed with a variety of inhibitors. The IC_{50} value is the concentration of inhibitor giving 50% inhibition of the enzyme activity assayed at pH 6.5 and 37°C with 1 mM 1-chloro-2,4,-dinitrobenzene and 2 mM GSH as substrates.

All the inhibitors studied, with the exception of cholic acid, were found to be capable of producing 50% inhibition of enzyme activity at concentrations above 125 μM. The group of organometal halides was the most potent of all the inhibitors used, the greatest inhibition being produced by tributyltin acetate.

Transferases B_1B_1, B_1B_2 and B_2B_2 did not respond equally to the effects of each inhibitor. Only small differences (up to 6-fold) in the degree of inhibition of transferase B_1B_1, compared to transferase B_2B_2, were found with cholic acid, lithocholic acid-3-sulphate, S-hexylglutathione and triphenyltin chloride. However, tributyltin acetate was at least 1000 times more effective at inhibiting the activity of transferase B_1B_1 compared to transferase B_2B_2. Hematin was about 30 times more effective with transferase B_1B_1 compared to transferase B_2B_2. Cibacron blue and bromosulphophthalein were also more effective inhibitors of B_1B_1 than B_2B_2 but produced less marked differences.

Table 3.3. Inhibition of basic GST activity.

IC_{50} values (μM) for human liver glutathione S-transferases B_1B_1, B_1B_2 and B_2B_2. The IC_{50} value is the concentration of inhibitor giving 50% inhibition of the enzyme activity assayed at pH 6.5, 37°C with 1 mM CDNB and 2 mM GSH as substrates.

	Subunit Composition		
Inhibitor	B_1B_1	B_1B_2	B_2B_2
	IC_{50} value (μM)		
Cibacron blue	2.5	7.8	24
Tributyltin acetate	<0.001	0.15	0.98
Triethyltin bromide	1.55	0.76	0.145
Triphenyltin chloride	0.3	0.7	1.5
Bromosulphophthalein	10.5	20	125
Hematin	1.5	3	40
S-Hexylglutathione	4.6	5.6	6.6
Cholic acid	1.15×10^3	2.4×10^3	4.1×10^3
Lithocholic acid-3-sulphate	6.6	28	36

Triethyltin bromide inhibited transferase B_2B_2 about 10 times more than transferase B_1B_1 and was the only compound tested which was more effective with transferase B_2B_2 than transferase B_1B_1.

All the IC_{50} values obtained for the B_1B_2 heterodimer were found to lie between those of the corresponding homodimers reflecting the contribution of the two functionally distinct subunits to the hybrid enzyme.

3.3 STRUCTURAL ANALYSIS OF HUMAN LIVER GST

a. Subunit composition of hepatic GST

The subunit composition of the human liver glutathione S-transferases was determined from their relative migration on SDS/polyacrylamide-gel electrophoresis (Fig. 3.7). The subunits from each of the purified enzymes B_1B_1, B_1B_2 and B_2B_2 migrated as a single band that ran between the Ya and Yb monomers from rat liver. Both subunits have an apparent Mr corresponding to 25 900.

The subunits from each of the purified glutathione S-transferase forms N_1 and N_2 migrated as a single band that ran between the Yb and Yc monomers from rat liver (Fig. 3.8). These subunits have an apparent Mr of 26 500. The subunit molecular weight of each purified GST is given in Table 3.5.

3.3b Isoelectric focusing of GST

The isoelectric point of each of the human glutathione S-transferases was determined using flat-bed isoelectric

Figure 3.7. SDS/polyacrylamide-gel electrophoresis of human liver GST.

The resolving gel contained 12% (w/v) polyacrylamide and 2.6% (w/v) NN'-methylenebisacrylamide. Samples were applied from left to right: lane 1, rat liver GST standards Ya (Mr 25 500), Yb (Mr (26 300) and Yc (Mr 27 500); lane 2, GST B_1B_1; lane 3, GST B_1B_2; lane 4, GST B_2B_2; lane 5, GST human basic (uncharacterised); lane 6, GST N_2; lane 7, rat liver Ya, Yb and Yc GST standards.

Note concerning GST in Lane 5

This GST was obtained from the DEAE-POOL 3 from a single liver and was resolved from GST N_2 using hydroxyapatite chromatography (Fig. 3.3a). It eluted as a minor peak at a sodium concentration of 254 mM, equivalent to that of the Basic GST forms. The molecular weight of the enzyme, Mr 25 900, is identical to that of the Basic GST forms. However, the isoelectric point pI 7.9 of the GST is distinct from the pI values of GST B_1B_1, B_1B_2 and B_2B_2 suggesting that it is a novel form of Basic enzyme. Further characterization of this GST could not be carried out due to the small amount of enzyme which was obtained.

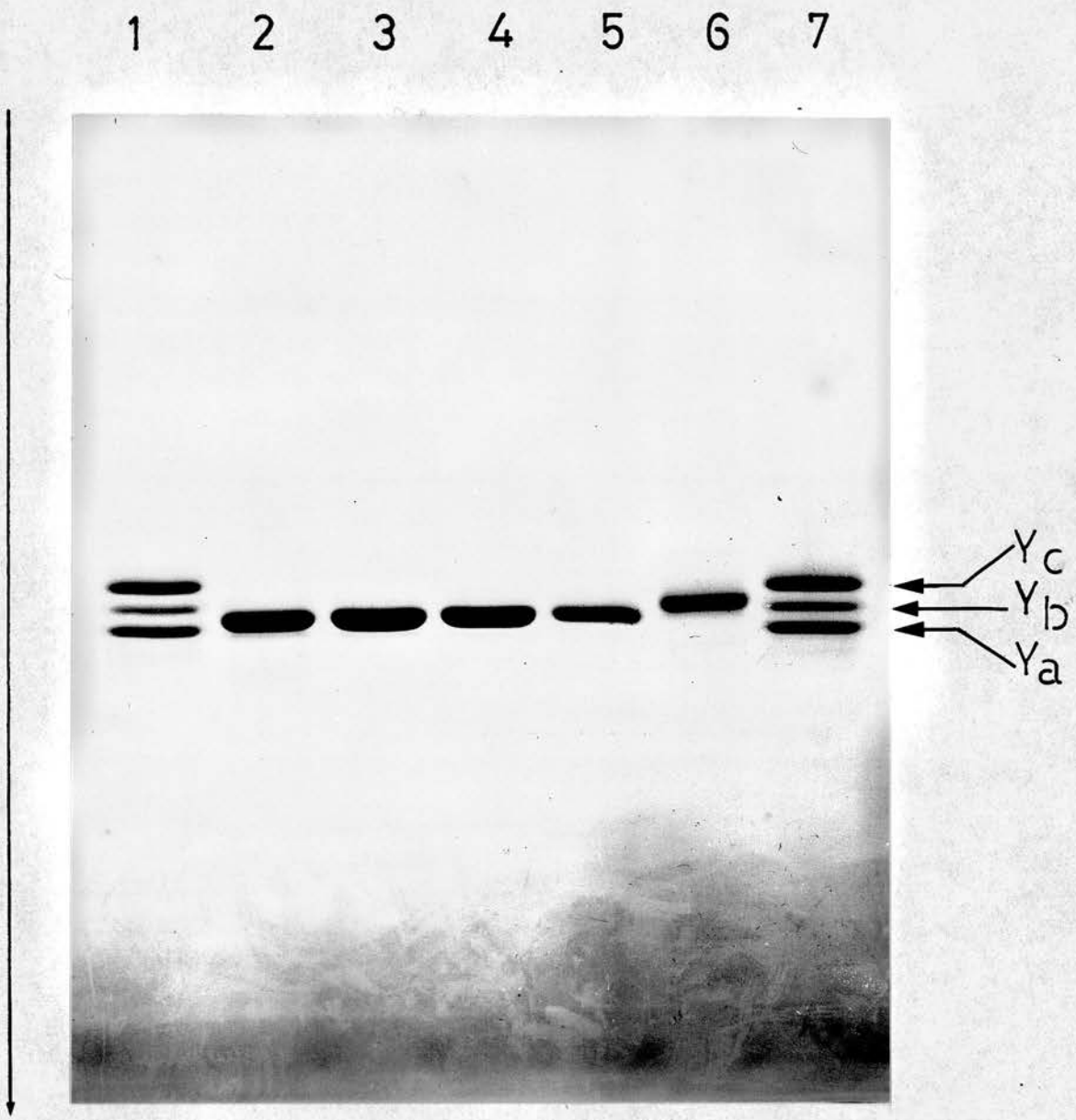


Figure 3.7

Figure 3.8. SDS/polyacrylamide-gel electrophoresis of GST N_1 and N_2 from human liver.

The resolving gel contained 12% (w/v) polyacrylamide and 2.6% (w/v) NN'-methylenebisacrylamide. Samples (approx. 10 μg protein) were applied from left to right: lane 1, GST N_1; lane 2, GST B_1B_2; lane 3, GST N_2; lane 4, GST λ ; lane 5, rat liver GST Ya, Yb and Yc standards. The dark staining below the protein bands in lanes 1 and 4 is due to the presence of Polybuffer and could be removed by precipitation using TCA (trichloroacetic acid).

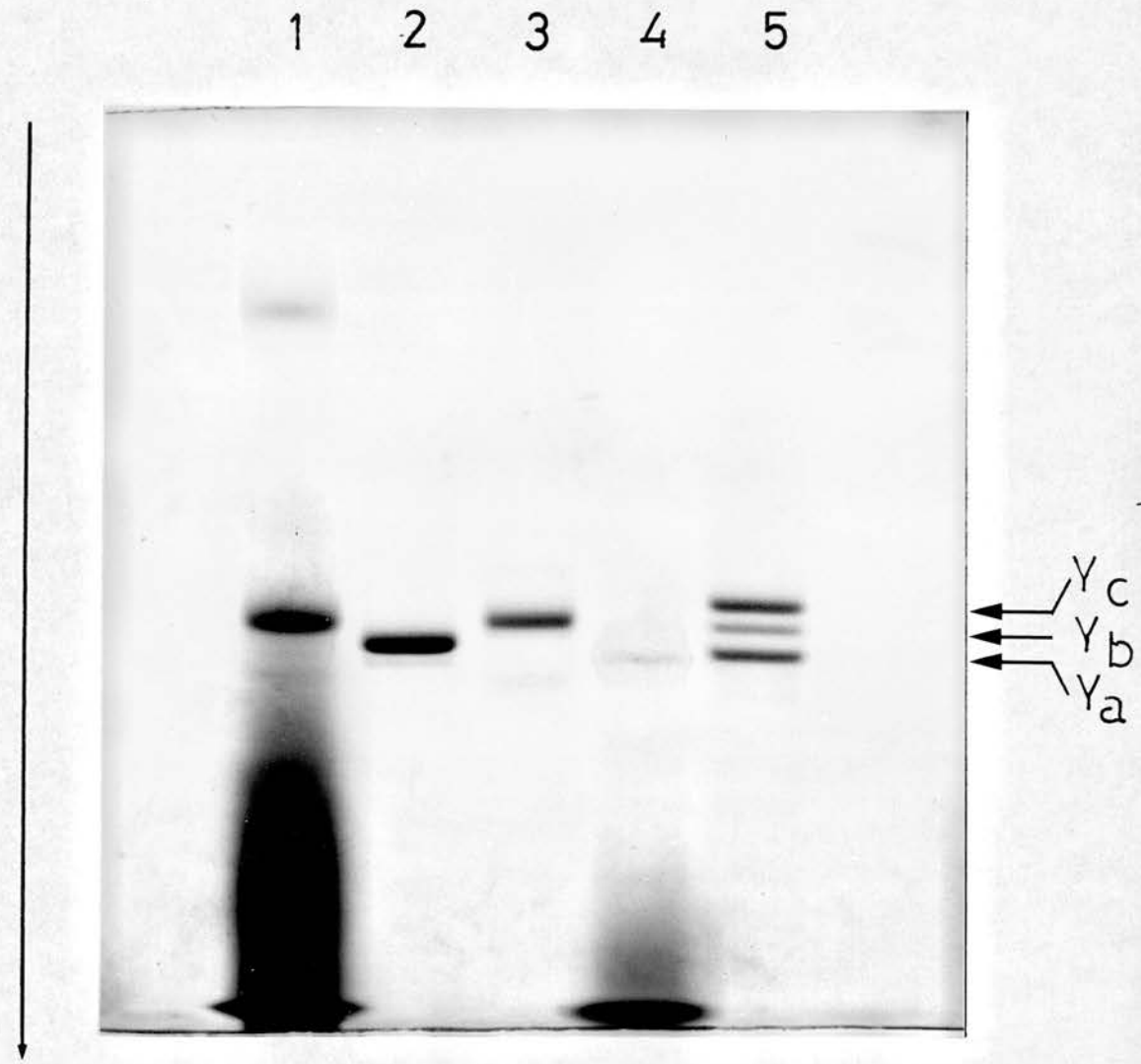


Figure 3.8

focusing of the native enzymes in the range pH 3.5 - 9.5 (Figs. 3.9 and 3.10). The isoelectric point values of the protein calibration standards are given in Table 3.4. Each focused protein appeared as a single major band with two faint 'less acidic' and one darker 'more acidic' bands closely ciated with it. This phenomenon was also observed to occur with the protein pI markers and is thought to be the result of oxidation of the samples. The pI of each major band of GST and their corresponding molecular weights are given in Table 3.5.

3.3c. Reversible dissociation of GST B_1B_2 subunits

After reversible dissociation of B_1B_2, three peaks of CDNB-GSH conjugating activity were resolved using DEAE-Sephadex chromatography (Fig. 3.11a). The first peak was not retained by the column; the second and third peaks were eluted by the salt gradient at Na^+ concentrations of 48 mM and 94 mM respectively. Native GST B_1B_2, which had not been subjected to guanidinium chloride treatment, was eluted in parallel from an identical DEAE-Sephadex column and served as a control (Fig. 3.11b).

Antisera raised against transferase B_1B_1 showed high cross-reactivity with the first and second peaks of activity and antisera raised against transferase B_2B_2 showed high cross-reactivity with the second and third peaks of activity.

Figure 3.9. Isoelectric focusing of human liver neutral GST.

This was performed using a broad-range gel (pH 3.5-9.5) in thin-layer 5% (w/v) polyacrylamide. The gel was loaded as follows: lane 1 and lane 5 contained protein pI calibration standards; lane 2, GST N_1; lane 3, GST N_2.

Isoelectric point values of protein calibration standards are presented in Table 3.4.

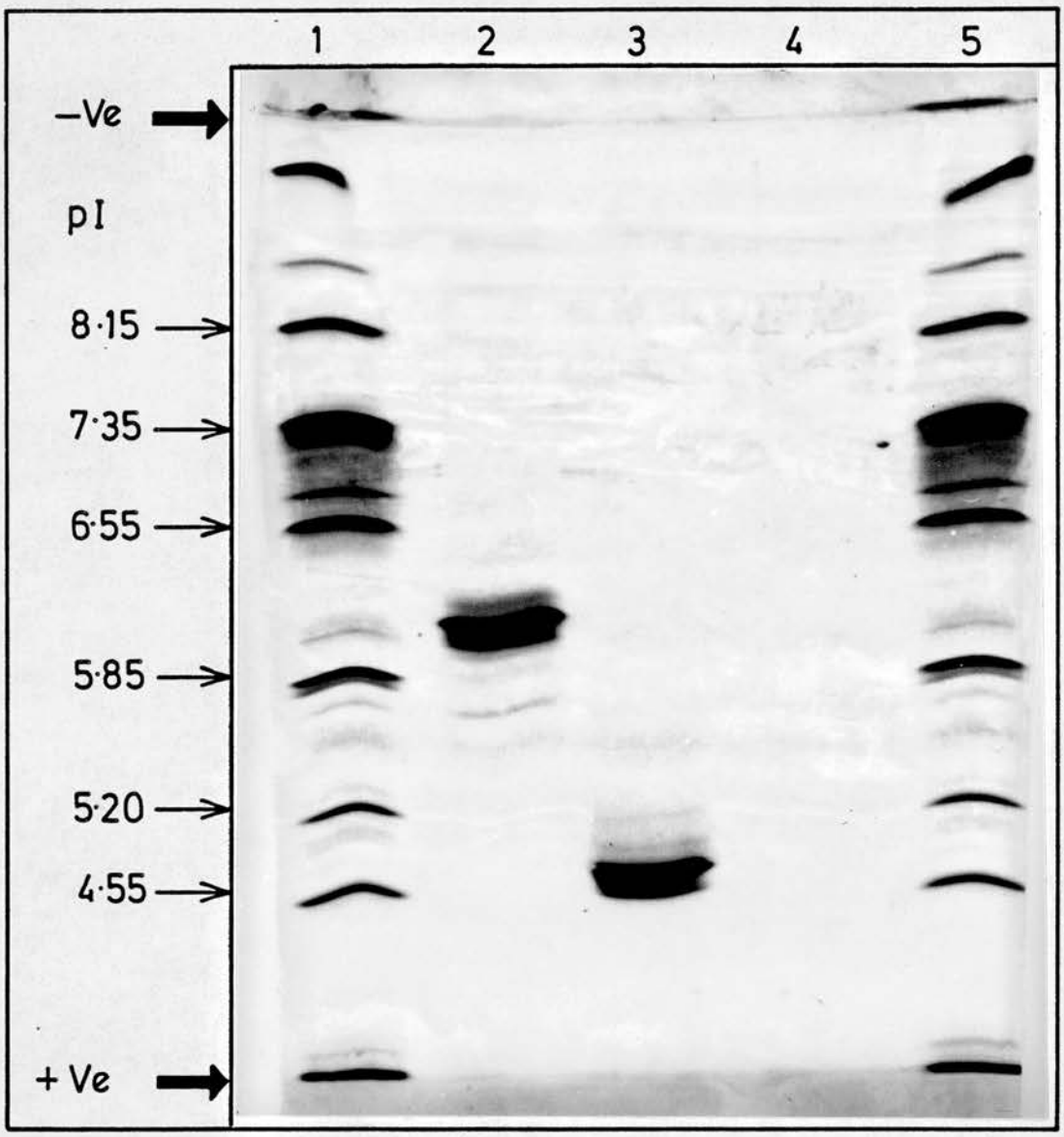


Figure 3.9

Figure 3.10. Isoelectric focusing of human liver basic GST.

This was performed using a broad-range gel (pH 3.5-9.5) in thin-layer 5% (w/v) polyacrylamide. The gel was loaded as follows: lane 1, protein pI calibration standards; lane 2, GST B_2B_2; lane 3, GST B_1B_2; lane 4, GST B_1B_1; lane 5, protein pI calibration standards.

Isoelectric point values of protein calibration standards are presented in Table 3.4.

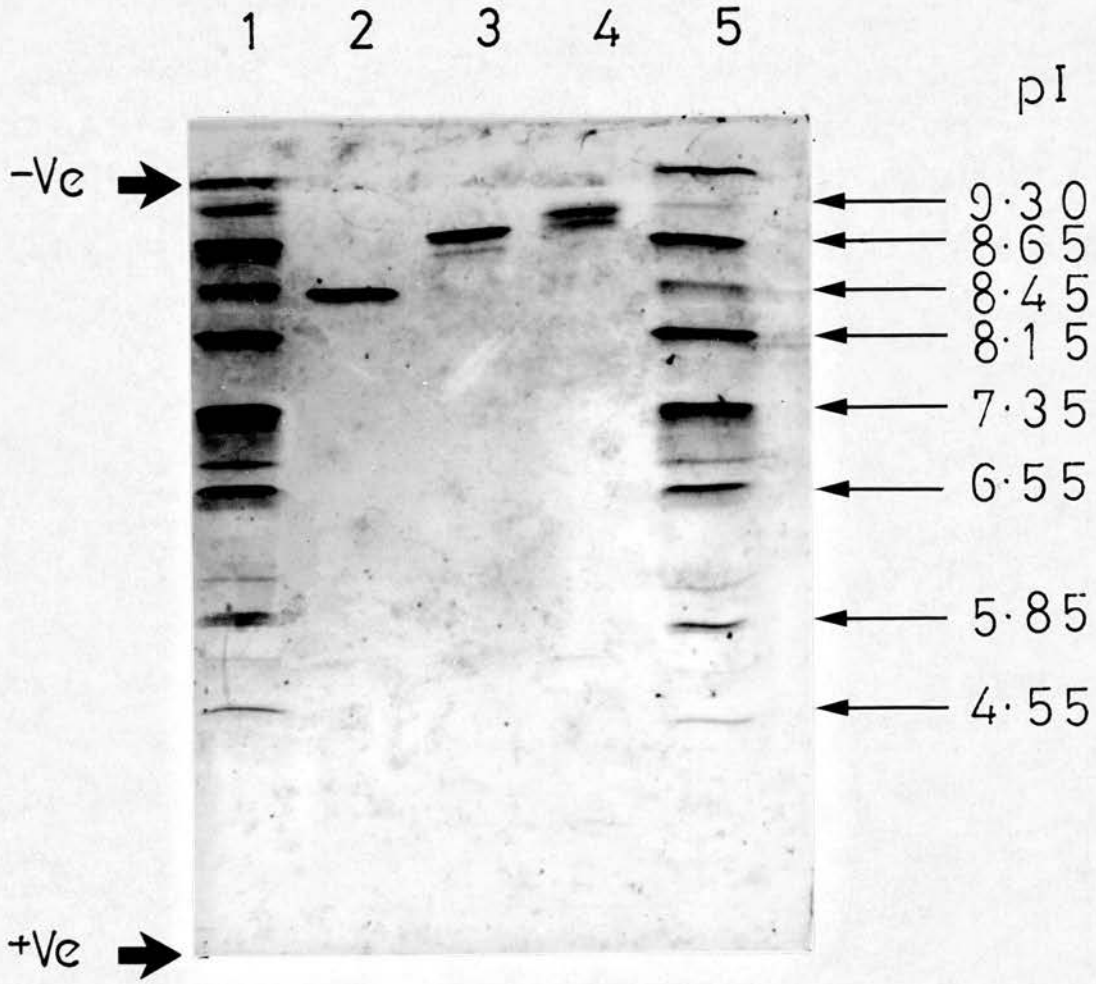


Figure 3.10

Protein	pI
trypsinogen	9.30
lentil lectin-basic band	8.65
lentil lectin-middle band	8.45
lentil lectin-acidic band	8.15
myoglobin-basic band	7.35
myoglobin-acidic band	6.85
human carbonic anhydrase B	6.55
bovine carbonic anhydrase B	5.85
β-lactoglobin A	5.20
soybean trypsin inhibitor	4.55
amyloglucosidase	3.50

Table 3.4. Isoelectric point values of protein calibration standards. Standards from Broad pI Calibration Kit supplied by Pharmacia Fine Chemicals, Uppsala, Sweden.

Transferase	Subunit Mr	Isoelectric point
B_1B_1	25 900	8.9
B_1B_2	25 900	8.75
B_2B_2	25 900	8.4
N_1	26 500	6.1
N_2	26 500	4.6
λ	24 800	4.8

Table 3.5. Electrophoretic properties of human liver GST

The subunit molecular weight of each enzyme was estimated using discontinuous SDS/polyacrylamide-gel electrophoresis. The resolving gel was 12% (w/v) polyacrylamide. Isoelectric point determination was performed in a 5% (w/v) flat bed polyacrylamide slab gel containing 0.2% (w/v) Ampholine.

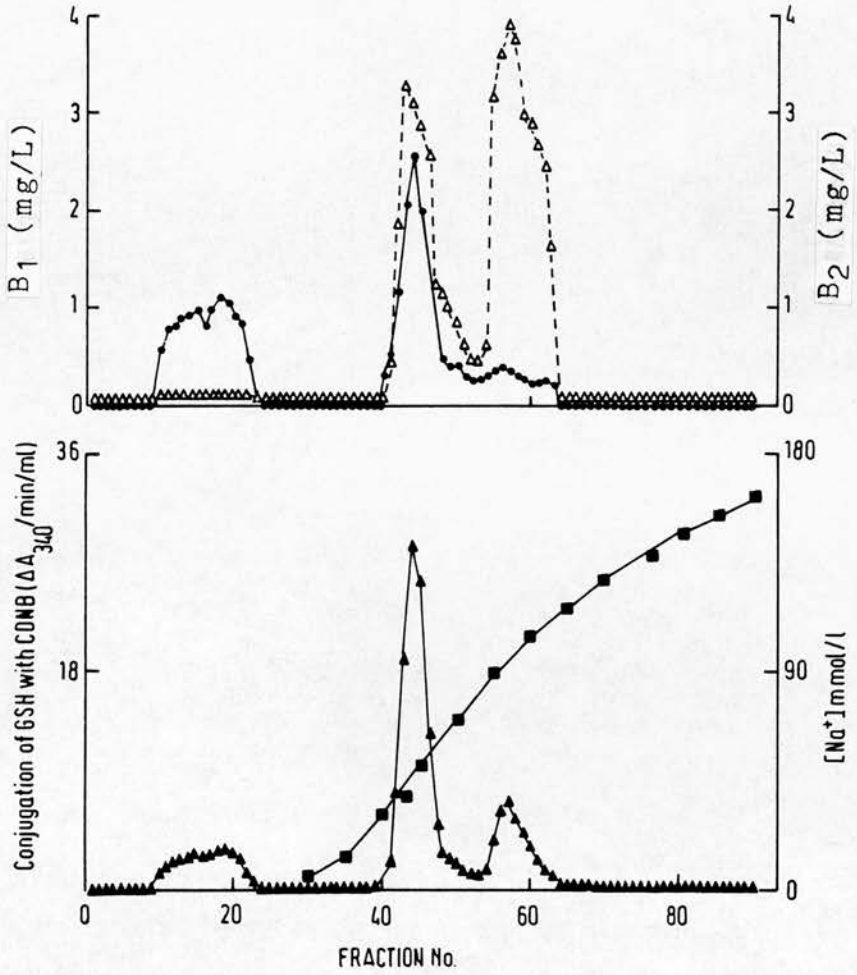


Figure 3.11. Separation of the products of reversible dissociation of GST B_1B_2.

(a) Transferase B_1B_2 (0.5 mg of protein) was incubated with 6M-guanidinium chloride, followed by dialysis against 20 mM Tris/HCl, pH 7.8. The non-diffusible material was applied to a column (2.2 cm x 21 cm) of DEAE-Sephadex that had been equilibrated with 20 mM Tris/HCl, pH 7.8, and a gradient of 0-250 mM NaCl was used to develop the column.

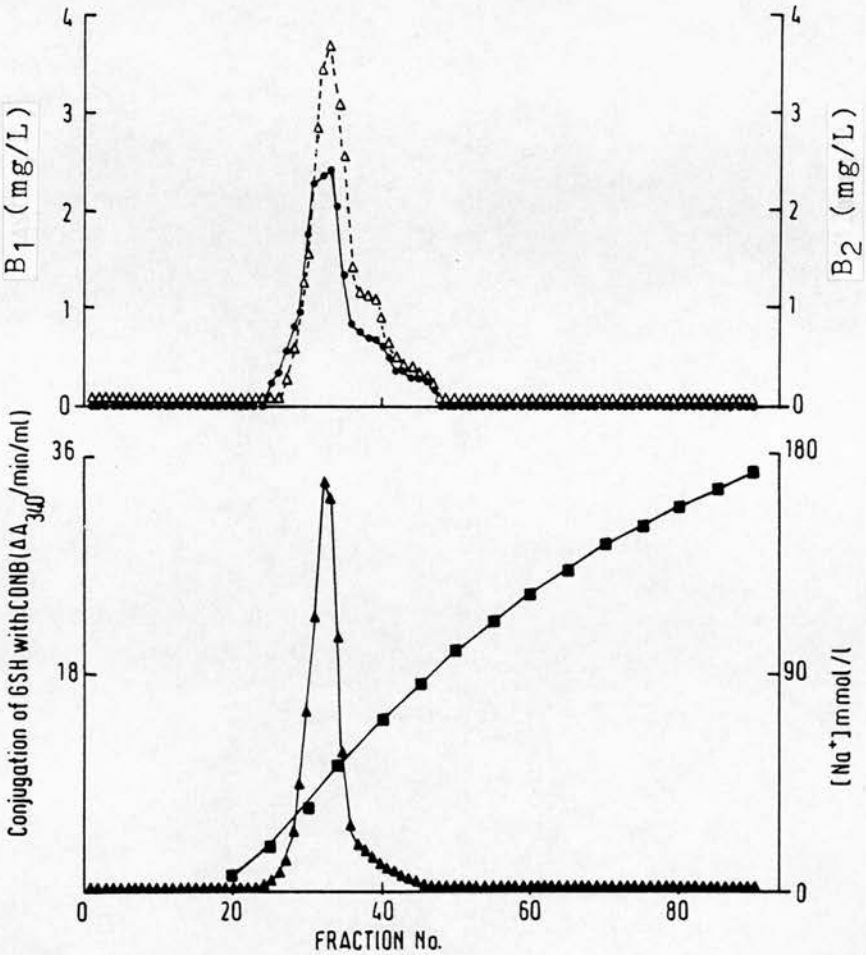


Figure 3.11 (contd.)

(b) A portion of transferase B_1B_2 that had not been incubated with 6M-guanidinium chloride was also applied to an identical DEAE-Sephadex column. The Na^+ concentration (■), transferase activity with CDNB (▲) and cross-reactivity with anti-B_1B_1 (●) and anti-B_2B_2 (△) sera were determined.

The three products of reversible dissociation of GST B_1B_2 that were separated on DEAE-Sephadex were individually subjected to CM-cellulose chromatography to help in their identification. Transferase B_2B_2 was eluted in the flow-through fractions (Figs. 3.12c) from CM-cellulose, whereas B_1B_1 was eluted as a single peak on the salt gradient at 45 mM (Fig. 3.12a). However, transferase B_1B_2 was eluted from CM-cellulose as two peaks on the salt gradient at Na^+ concentrations 25 mM and 36 mM (Fig. 3.12b). Each of these, when separately re-applied to identical columns after storage (4°C for 30 days), co-eluted from CM-cellulose as a single peak at a position corresponding to the form of the enzyme that was originally eluted at the higher Na^+ concentration (Figs. 3.12d, 3.12e).

Portions of the re-associated transferases B_1B_1, B_1B_2 and B_2B_2 were subjected to isoelectric focusing and resulted in the resolution of major bands of protein with pI values of 8.9, 8.75 and 8.4 respectively, corresponding to the pI values of their native counterparts.

3.3d. Reversible dissociation of GST N_1 subunits

When GST N_1 that had been subjected to reversible dissociation was applied to DEAE-Sephadex, it was eluted on the salt gradient as a single peak of activity at an Na^+ concentration of 49 mM.

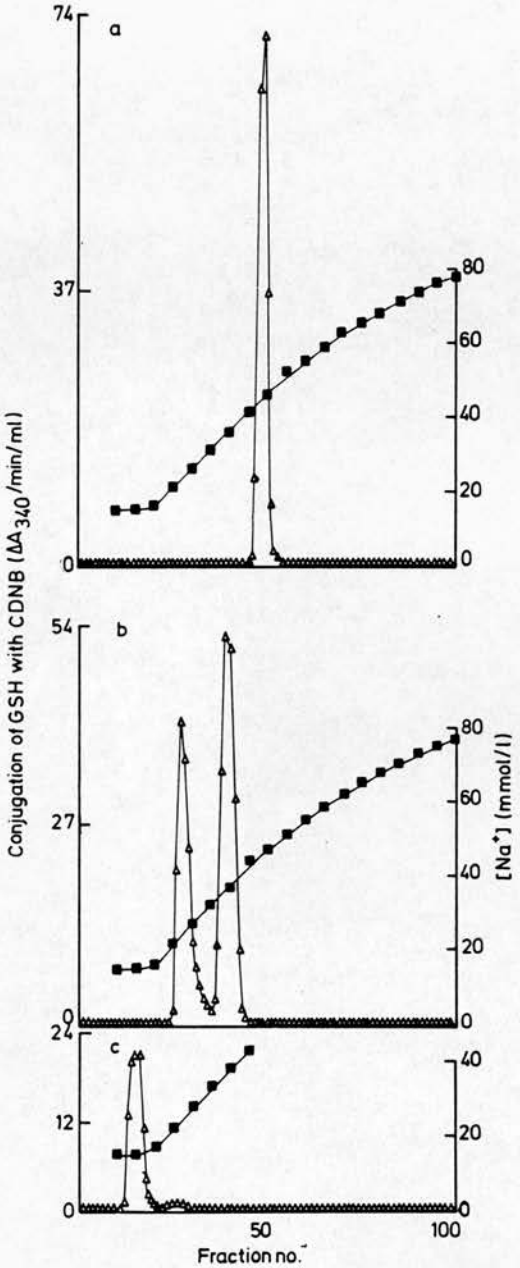


Figure 3.12. CM-cellulose chromatography of the re-associated products obtained from GST B_1B_2.

Transferases B_1B_1, B_1B_2 and B_2B_2, which were obtained by reversible dissociation of transferase B_1B_2 were resolved by DEAE-Sephadex chromatography as described in the text. Portions (25-60 μg of protein) of these enzymes were dialysed against 2 x 1 litre of 10 mM sodium phosphate, pH 6.7, before being applied to identical columns (2.2 cm x 24 cm) equilibrated with the same buffer. A 0-100 mM NaCl gradient was used to develop these columns. Fractions (4.0 ml) were collected and the Na^+ concentration (■) and transferase activity with CDNB (△) were measured. (a) B_1B_1; (b) B_1B_2; (c) B_2B_2.

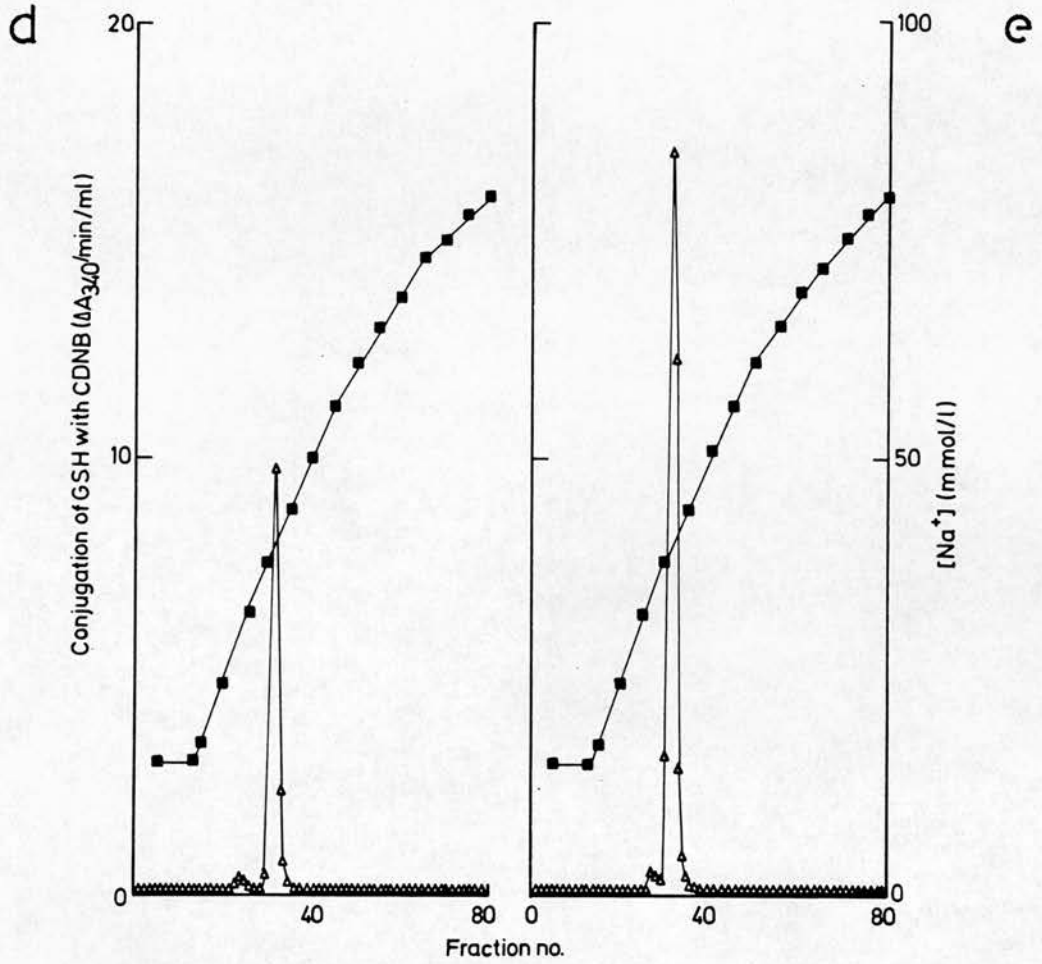


Figure 3.12 (contd.)

This procedure resulted in the resolution of two peaks of enzyme activity from transferase B_1B_2. Each of these was re-subjected to chromatography and analysed by using the conditions described. Fractions (3.8 ml) were collected. (d) First peak from profile shown in (b); (e) second peak from profile shown in (b).

3.3e. Immunochemical analysis of human hepatic GST by the 'Western' blot technique

The immunochemical relationship between the human hepatic transferases was examined by using the 'Western' blot technique. The transferase subunits were resolved by discontinuous SDS/polyacrylamide-gel electrophoresis and then transferred electrophoretically onto sheets of nitrocellulose. Each sheet was incubated with an antiserum raised to a purified form of human liver transferase. The subunits that cross-reacted with the antisera were detected by staining with a peroxidase-linked second antibody. The results of the 'Western' blots are given in Fig. 3.13 and Table 3.6.

All the proteins which were analysed cross-reacted with their corresponding antisera. These samples did not stain with equal density because the antisera were used at different dilutions to compensate for variations in their affinity. The more intensely stained samples, transferases N_1 and N_2, revealed polypeptides of molecular weight approx. Mr 24 500. These are thought to be degradation products of the enzymes rather than contaminants since the two enzymes were obtained by different methods of purification.

Transferases B_1B_1, B_1B_2 and B_2B_2 all cross-reacted with anti-(transferase B_1B_1) and anti-(transferase B_2B_2) antisera, but transferases N_1 and N_2 did not cross-react with these antisera. Conversely, transferases N_1 and N_2

Figure 3.13. 'Western' blot analysis of human liver GST.

Portions (approx. 10 μg) of purified human liver transferases were subjected to SDS/polyacrylamide-gel electrophoresis and then transferred in replica onto sheets of nitrocellulose paper. The nitrocellulose papers were incubated with (a) anti-(transferase B_1B_1), (b) anti-(transferase B_2B_2), (c) anti-(transferase N_1) and (d) anti-(transferase N_2) antisera. The blots probed with these 4 sets of antisera are shown on pages 146-149. The polypeptides that cross-reacted were detected with the Bio-Rad goat anti-(rabbit IgG) antibody-horseradish-peroxidase conjugate immunoblot assay kit. The orientations of each nitrocellulose sheet depicted, (a)-(d), are identical as are the order and loading of each sample of protein. The samples in each lane are as follows: lane 1, transferase B_1B_1; lane 2, transferase B_1B_2; lane 3, transferase B_2B_2; lane 4, transferase N_1; lane 5, transferase N_2. The origin of the SDS/polyacrylamide-gel is at the top of the nitrocellulose immune-replica.

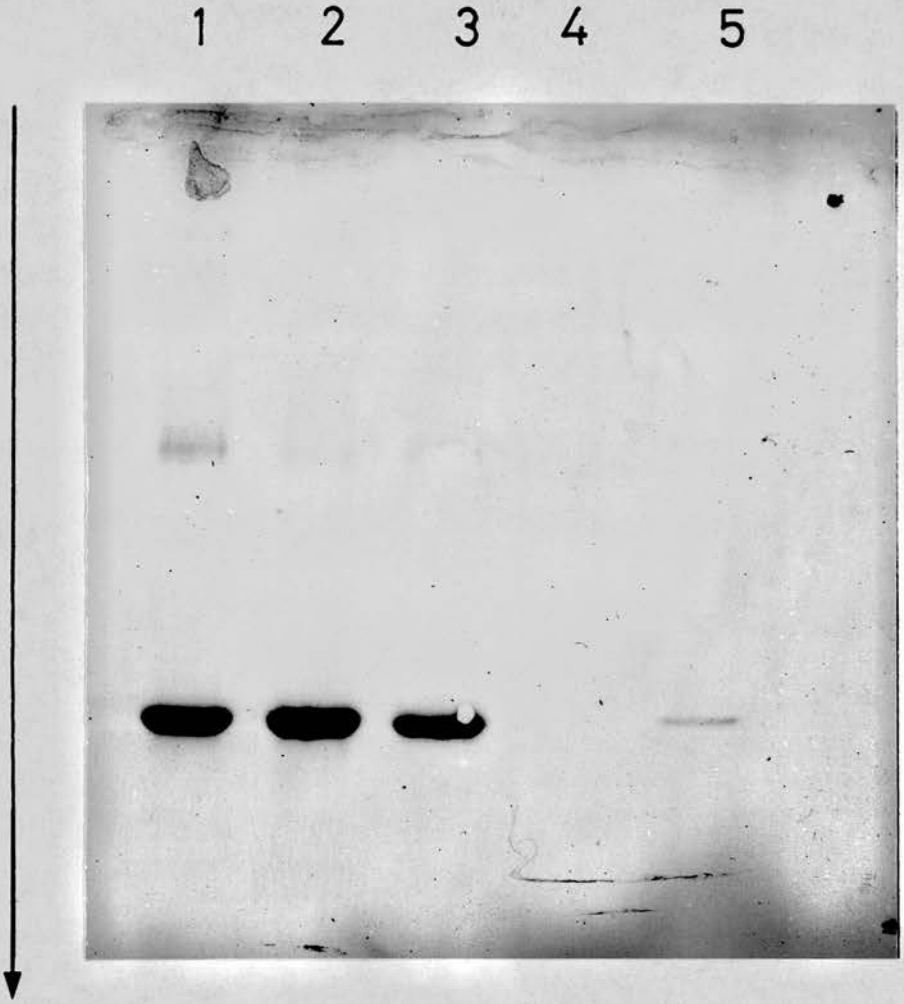


Figure 3.13a. 'Western' blot analysis of human liver GST using anti-(transferase B_1B_1) antisera: lane 1, GST B_1B_1; lane 2, GST B_1B_2; lane 3, GST B_2B_2; lane 4, GST N_1; lane 5, GST N_2.

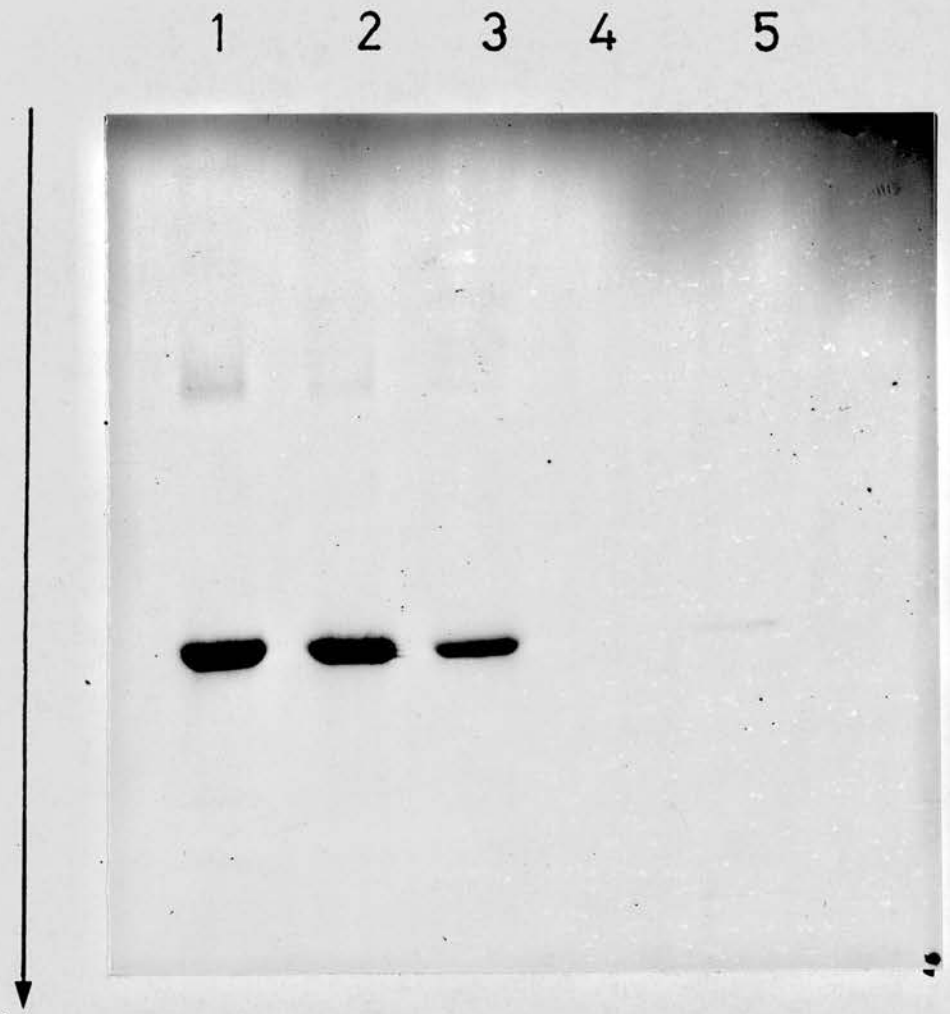


Figure 3.13b. 'Western' blot analysis of human liver GST using anti-(transferase B_2B_2) antisera: lane 1, GST B_1B_1; lane 2, GST B_1B_2; lane 3, GST B_2B_2; lane 4, GST N_1; lane 5, GST N_2.

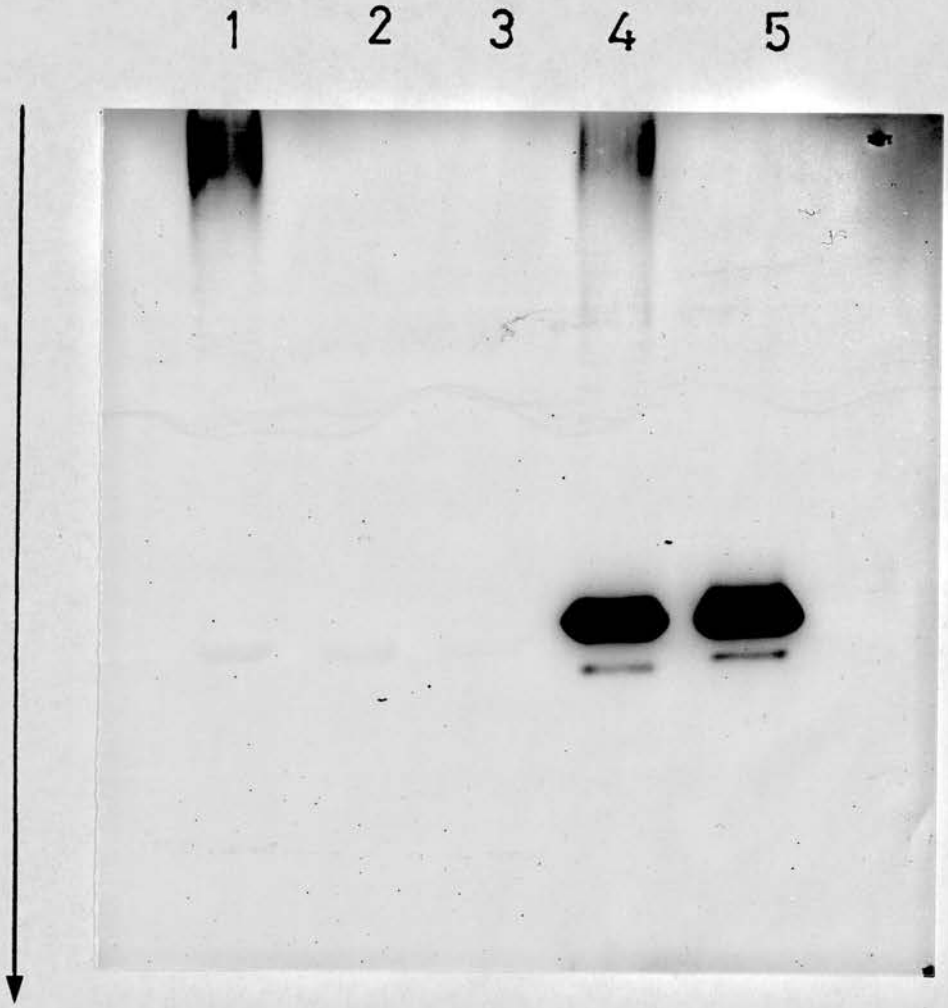


Figure 3.13c. 'Western' blot analysis of human liver GST using anti-(transferase N_1) antisera: lane 1, GST B_1B_1; lane 2, GST B_1B_2; lane 3, GST B_2B_2; lane 4, GST N_1; lane 5, GST N_2.

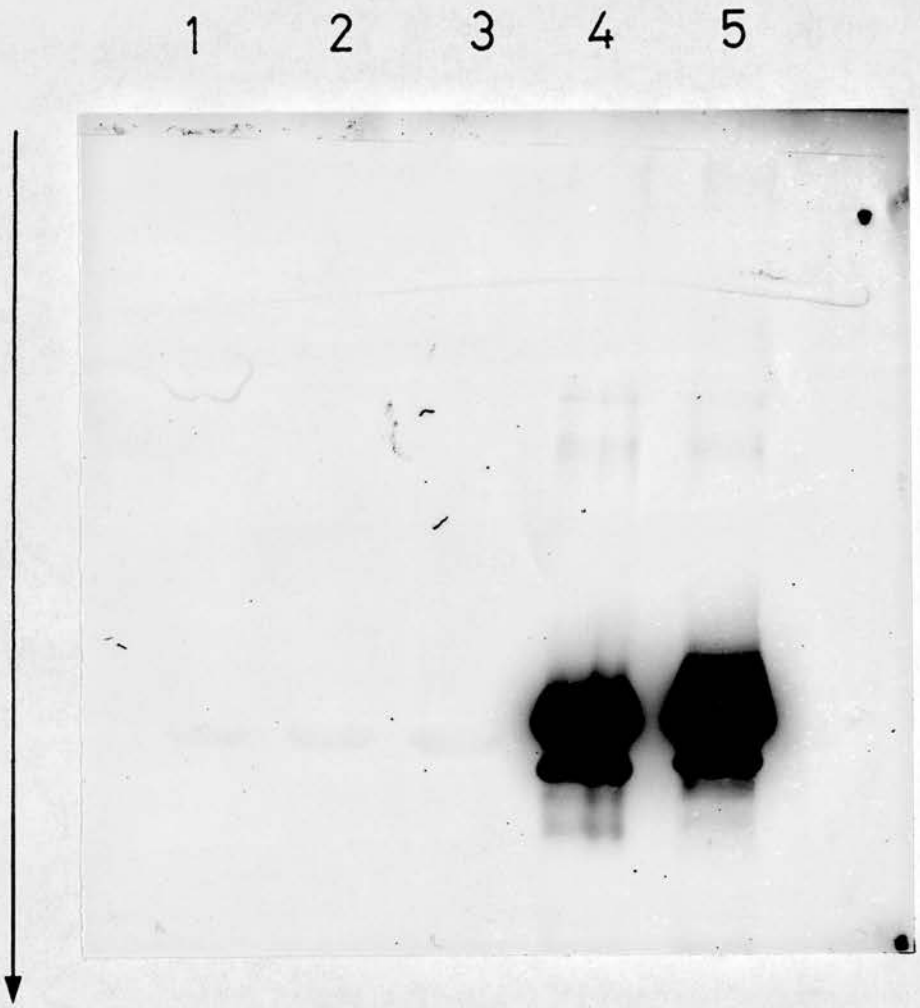


Figure 3.13d. 'Western' blot analysis of human liver GST using anti-(transferase N_2) antisera: lane 1, GST B_1B_1; lane 2, GST B_1B_2; lane 3, GST B_2B_2; lane 4, GST N_1; lane 5, GST N_2.

Transferase	Subunit Mr	Cross-reaction between transferase and antiserum			
		Antiserum			
		Anti-B_1B_1	Anti-B_2B_2	Anti-N_1	Anti-N_2
B_1B_1	25 900	+	+	-	-
B_1B_2	25 900	+	+	-	-
B_2B_2	25 900	+	+	-	-
N_1	26 500	-	-	+	+
N_2	26 500	-	-	+	+

Table 3.6. Cross-reactivity of GST using 'Western' blot analysis.

Portions (10 μg) of human glutathione S-transferases were subjected to SDS/polyacrylamide-gel electrophoresis and transferred electrophoretically to nitrocellulose paper by the method of Towbin et al. (1979). The cross- reactivity of each protein with several anti-(glutathione S-transferase) antisera was examined as described in section 2.7b.

A positive result (+) indicates that the protein sample cross-reacted strongly with the antiserum. This appeared as a dense stain on the nitrocellulose paper using the Bio-Rad goat anti-(rabbit IgG) antibody-horseradish-peroxidase conjugate immunoblot assay kit.

cross-reacted with anti-(transferase N_1) and anti-(transferase N_2) antisera but transferases B_1B_1, B_1B_2 and B_2B_2 did not cross-react with these antisera.

The immunochemical data presented allow the enzymes to be divided into two distinct groups, i.e. those enzymes that cross-react with the anti-(transferase B_1B_1 or B_2B_2) antisera and those enzymes that cross-react with the anti-(transferase N_1 or N_2) antisera. These groups correspond to those based on the molecular weight of these enzymes.

3.3f. Radioimmunoassay of human hepatic GST

Radioimmunoassay was carried out on portions of GST using antisera raised to GST B_1B_1 and B_2B_2. Using this method, antisera raised against GST B_1B_1 cross-reacted with B_1B_1 and B_1B_2 but not with B_2B_2. Conversely, antisera raised against GST B_2B_2 cross-reacted in the radioimmunoassay with B_2B_2 and B_1B_2 but not with B_1B_1. These antisera did not cross react with GST N_1 or GST λ (Table 3.7).

3.4 EVIDENCE THAT B_1 and B_2 SUBUNITS REPRESENT SEPARATE GENE PRODUCTS

a. Amino acid compositions

The amino acid compositions of transferases B_1B_1, B_1B_2, B_2B_2 and N_1 were determined. These were compared with the compositions of human enzymes which had been published previously to establish if the enzymes described in this work are closely related or identical to those described previously. The results were also analysed to determine if the B_1 and B_2 subunits are derived from

Transferase	Relative cross-reactivity (%)	
	Anti-B_1B_1	Anti-B_2B_2
B_1B_1	100	0.2
B_1B_2	55	58
B_2B_2	0.3	100
N_1	<0.1	<0.1
λ	<0.1	<0.1

Table 3.7. Immunochemical analysis of human GST by radioimmunoassay.

Full experimental details are given in the text. The relative cross-reactivity is defined as that quantity of GST that is required to produce 50% displacement of bound ligand when compared with the most immunoreactive protein.

totally different genes, closely related genes (same gene family) or the same gene. Table 3.8 lists the amino acid compositions which were determined from 24 h HCl hydrolysis of each enzyme sample.

Amino acid	Amount of amino acid as % of total recovered			
	B_1B_1	B_1B_2	B_2B_2	N_1
Lys	11.5	10.5	10.8	10.6
His	1.5	1.2	1.3	2.5
Arg	5.2	5.3	5.1	4.8
Asx	8.4	8.2	8.4	11.4
Thr	2.3	1.8	1.9	3.2
Ser	5.7	5.0	6.3	5.0
Glx	11.5	15.1	13.4	6.5
Pro	5.1	4.8	4.6	5.3
Gly	6.1	5.1	5.2	5.4
Ala	7.6	7.4	6.4	4.7
Val	4.4	4.3	3.9	3.5
Met	2.9	3.0	3.4	3.6
Ile	5.5	6.2	6.9	7.1
Leu	14.3	13.5	13.6	13.6
Tyr	4.0	4.3	4.4	6.2
Phe	4.0	4.3	4.4	6.6

Table 3.8. Amino acid compositions of GST enzymes.

Samples (0.5 mg of protein) were hydrolysed in 6M HCl for 24 h. The results were obtained in μmol of amino acid recovered. These results were used to calculate the percentage amino acid composition for each protein. The recoveries of cysteine and tryptophan were not determined and have been excluded from the calculations. The compositions were not corrected for the increased or decreased recoveries of Ser, Thr, Val and Ile.

3.4b. Peptide 'mapping' experiments

Peptide 'mapping' is a technique for detecting differences in the primary structure of proteins which are highly homologous (>80%). The method described by Ambler (1963) involves complete proteolytic digestion of protein, followed by separation of fragments, according to charge by high-voltage paper electrophoresis in one or two dimensions. Hayes (1983) has successfully used this method to demonstrate the different peptides which are produced by tryptic digestion of the rat GST A (Yb_1Yb_1) and D (Yb_2Yb_2).

The peptides produced by tryptic digestion of transferases B_1B_1, B_1B_2 and B_2B_2 were subjected to high-voltage paper electrophoresis at pH 6.5 on a single sheet of chromatography paper. This procedure separated the peptides of each transferase into three zones of mobility: basic, neutral and acidic. These zones were identified by running in parallel a second, smaller, sample of each enzyme. The separated peptides from this second sample were stained.

The tryptic peptides from each enzyme were incompletely resolved by this first dimension electrophoresis and it was not possible to detect differences in the patterns produced by each enzyme (Fig. 3.14). However, the sample from transferase B_1B_2 gave rise to a very acidic peptide which ran close to the anode and was not present in either of the other samples. This peptide was not included in the second dimension of the peptide 'mapping'

Figure 3.14. One dimensional 'maps' of tryptic digests of B_1B_1, B_1B_2 and B_2B_2.

Portions of transferase B_1B_1, B_1B_2 and B_2B_2 (2.0 mg of protein) were digested at 37°C with trypsin (50 µg of proteinase). After 18 h at 37°C the digests were freeze-dried. The freeze-dried digestion products were dissolved in 100 µl of 0.1 M NH_3 and applied to Whatman 3 MM chromatography paper from left to right as follows: 1, 0.1 mg of tryptic digest of B_2B_2; 2, 0.1 mg of tryptic digest of B_1B_2; 3, 0.1 mg of tryptic digest of B_1B_1. The unlabelled lanes contained the 'Wondermix' amino acid and dye standards. The origin is marked; the cathode is at the top of the figure and the anode at the bottom. The peptides were divided into three groups which were designated the basic, neutral and acidic peptides according to their electrophoretic mobility at pH 6.5. The remaining portion (approx. 1.9 mg of protein) of each tryptic digest was run in parallel but it is not included in the figure.

The electrophoresis paper was divided into portions to permit peptide separation in a second dimension as described in Section 2.8 and figure 2.2.

Abbreviations: ATP, acidic tryptic peptides;

BTP, basic tryptic peptides and NTP, neutral tryptic peptides

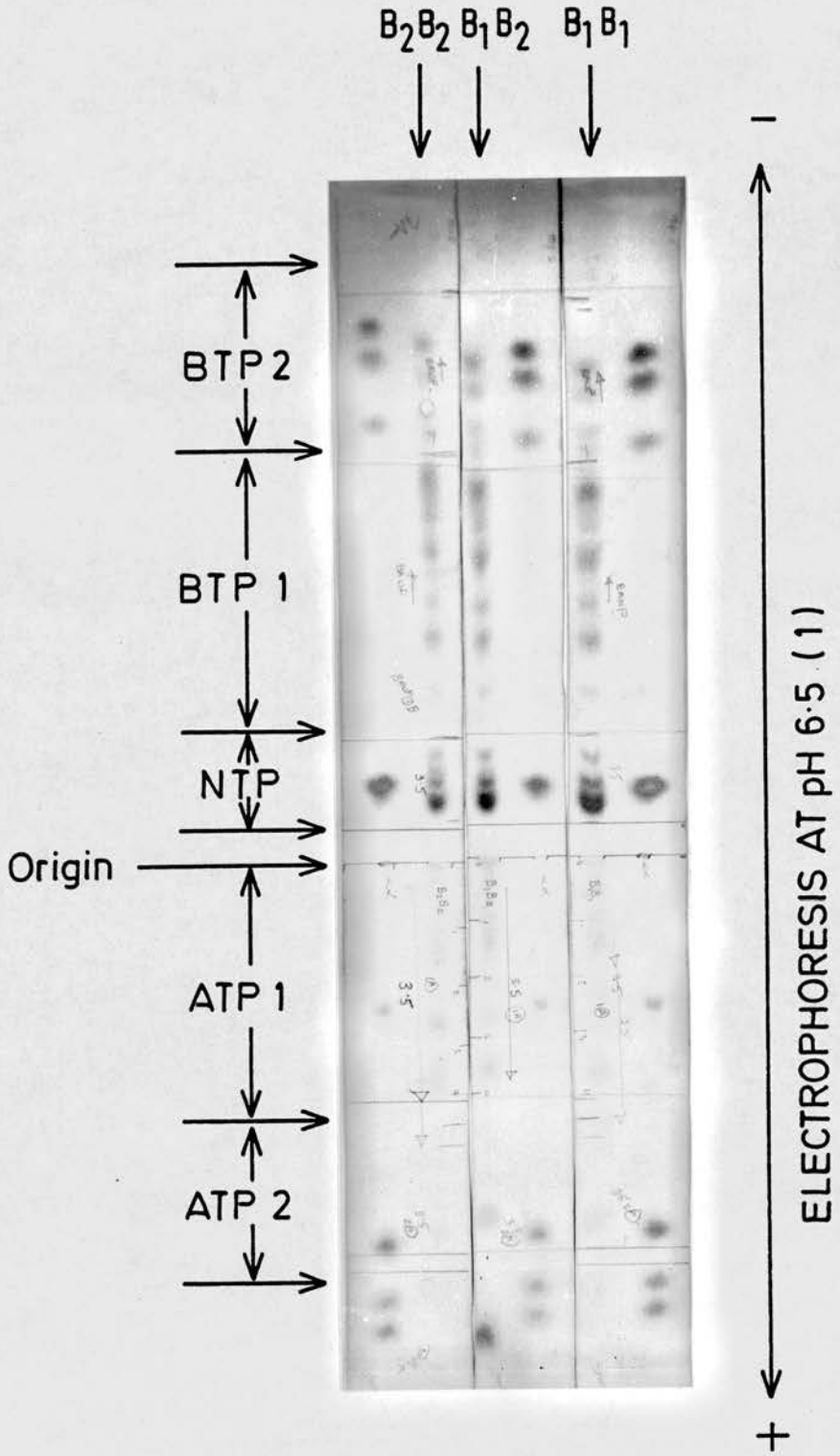

B_2B_2 B_1B_2 B_1B_1
BTP 2
BTP 1
NTP
Origin
ATP 1
ATP 2
ELECTROPHORESIS AT pH 6·5 (1)
−
+

Figure 3.14

analysis but was cut-out and eluted from the electrophoresis paper. Amino acid analysis of the acidic peptide demonstrated that it was glutathione.

3.4bi. Acidic tryptic peptides from B_1B_1, B_1B_2 and B_2B_2

Two dimensional peptide 'maps' of the acidic peptides of transferases B_1B_1, B_1B_2 and B_2B_2 were constructed by high-voltage paper electrophoresis at pH 6.5 in the first dimension and at pH 3.5 in the second dimension.

A diagrammatic representation of the acidic peptide 'maps' following the second dimension electrophoresis at pH 3.5 is shown in Fig. 3.15. Seven peptides (shaded black) were demonstrated to be common to all three proteins. One peptide (no shading) was found to be present only in transferases B_1B_1 and B_1B_2 while another peptide (hatched shading) was only present in transferases B_1B_2 and B_2B_2.

3.4ii. Neutral tryptic peptides from B_1B_1, B_1B_2 and B_2B_2

The neutral tryptic peptides were poorly resolved and remained close to the origin following electrophoresis at pH 6.5. However, an improved separation was achieved by further electrophoresis at pH 3.5. Finally, descending chromatography in a second dimension produced a 'map' of well resolved peptides from each enzyme.

A diagrammatic representation of the neutral tryptic 'maps' of transferases B_1B_1, B_1B_2 and B_2B_2 is shown in Fig. 3.16. At least 10 peptides (black shading) appear

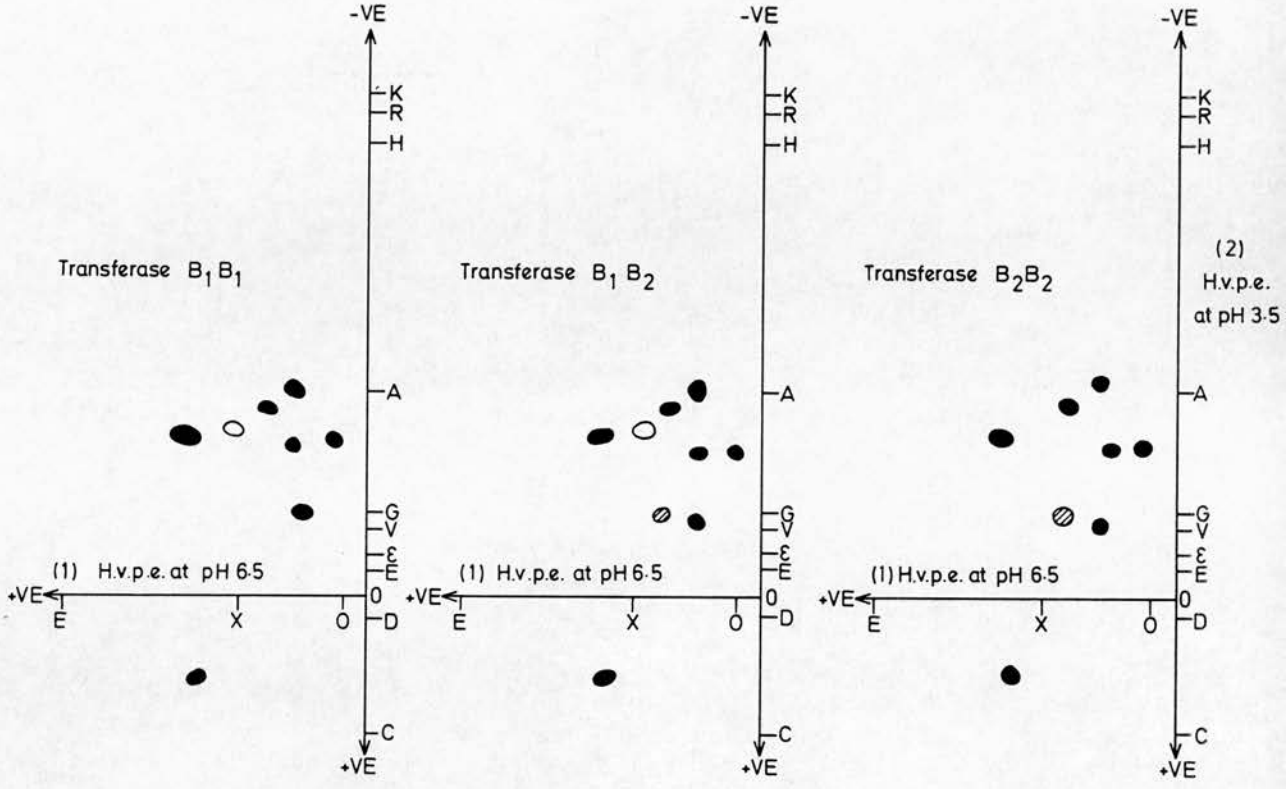


Figure 3.15. Tryptic 'map' of the acidic peptides from basic GST enzymes.

The tryptic digests of portions (2.0 mg of protein) of B_1B_1, B_1B_2 and B_2B_2 were prepared. The petides were separated by high-voltage paper electrophoresis at pH 6.5 in the first dimension and the acidic peptides were cut out of the paper as indicated in Fig. 2.2. These peptides were separated in the second dimension by high-voltage paper electrophoresis at pH 3.5. The stained peptide spots were traced to produce the diagram. No tryptophan-containing peptides were detected. The definition of the symbols is given in Fig. 2.1.

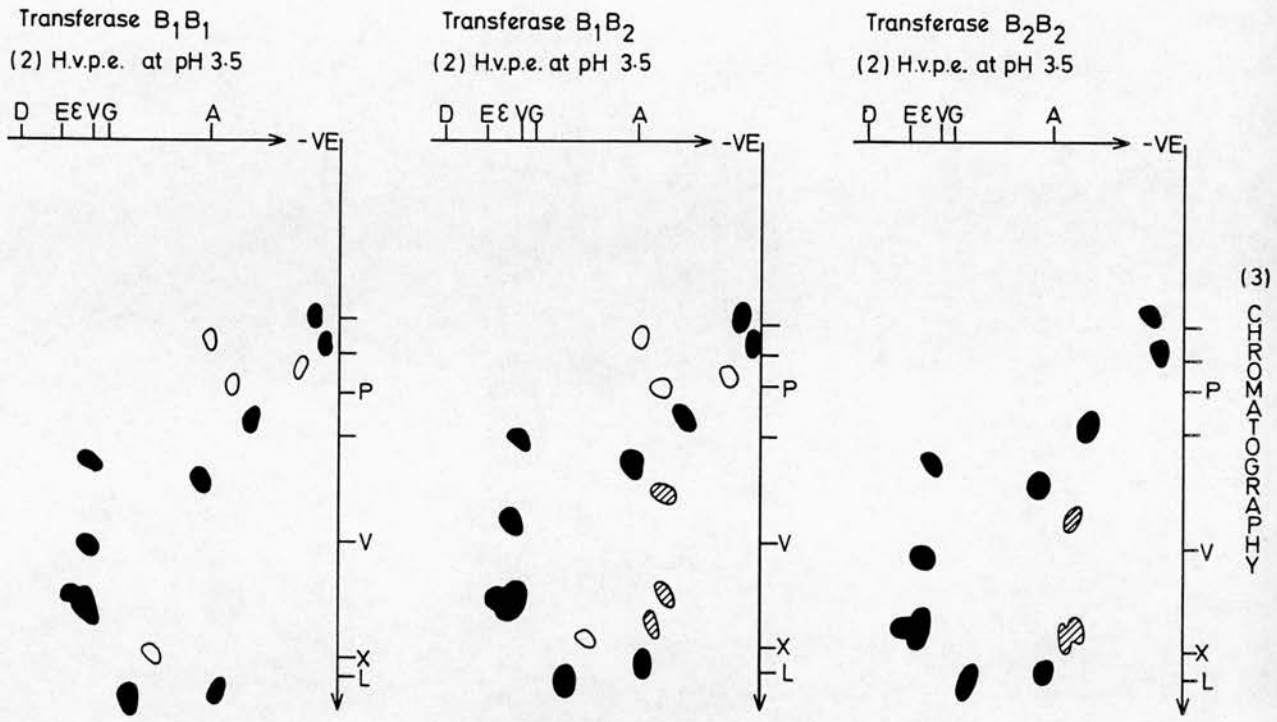


Figure 3.16. Tryptic 'map' of the neutral peptides from basic GST enzymes.

The tryptic digests of portions (2.0 mg of protein) of B_1B_1, B_1B_2 and B_2B_2 were separated by high-voltage paper electrophoresis at pH 6.5 in the first dimension and the basic peptides were cut out of the paper as indicated in Fig. 2.2. These peptides were further separated in the same dimension by high-voltage paper electrophoresis at pH 3.5. The second dimension separation was performed using descending chromatography using butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) for 16 h. The stained peptide spots were traced to produce the diagram. No tryptophan-containing peptides were detected. The definition of the symbols is given in Fig. 2.1.

to be common to all of these enzymes. Transferases B_1B_1 and B_1B_2 each have four peptides (no shading) which are common to them and not transferase B_2B_2 and transferases B_1B_2 and B_2B_2 have 3 peptides (hatched shading) which are common to them and not transferase B_1B_1.

3.4biii. Basic peptides from transferases B_1B_1, B_1B_2 and B_2B_2

Two dimensional peptide 'maps' of the basic tryptic peptides were constructed by high-voltage paper electrophoresis at pH 6.5 in the first dimension and descending chromatography in the second dimension.

The basic peptides from transferases B_1B_1, B_1B_2 and B_2B_2 were well resolved by the electrophoresis at pH 6.5, however, no differences between the peptide pattern from each enzyme were apparent. Following descending chromatography in the second dimension differences in the peptide maps were easily identified.

The two dimensional 'map' of the basic peptides is shown diagrammatically in Fig. 3.17. At least 19 tryptic peptides (black shading) appear to be common to all three enzymes while at least two peptides (no shading) are only present in B_1B_1 and B_1B_2 but not B_2B_2.

The results of the tryptic peptide maps shown in Figs. 3.15 - 3.17 demonstrate that a considerable structural homology exists between transferases B_1B_1, B_1B_2 and B_2B_2. The total number of tryptic peptides from B_1B_1,

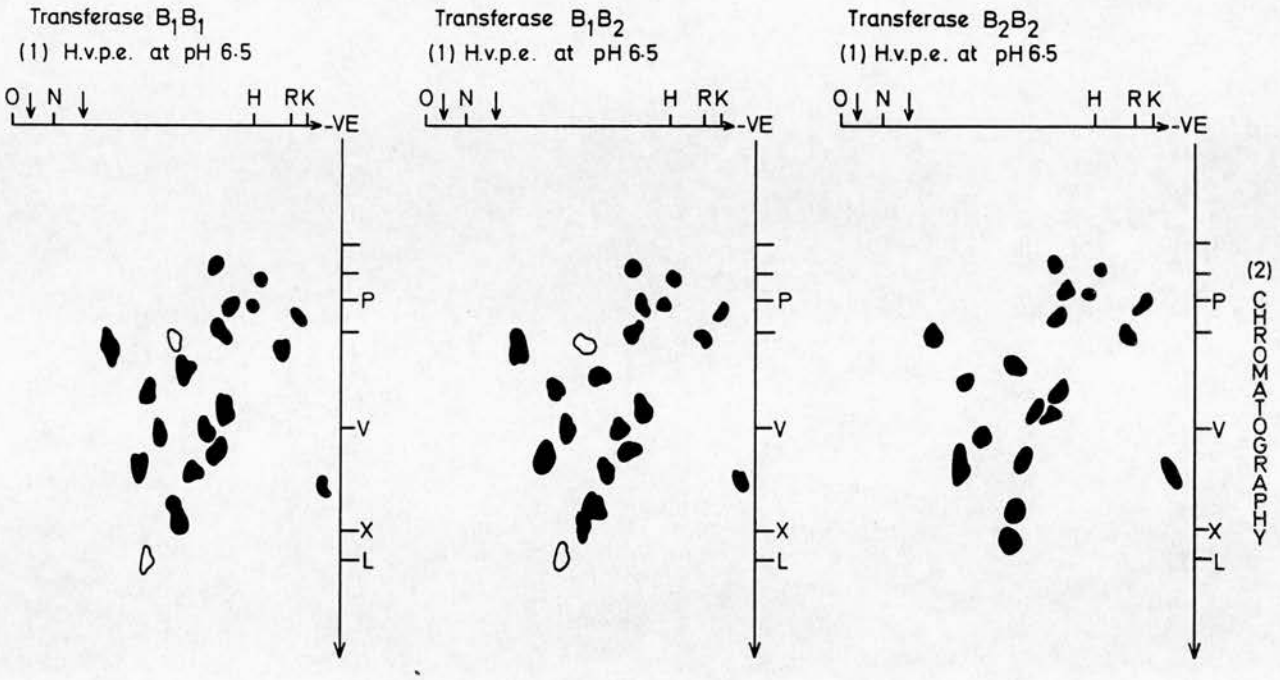


Figure 3.17. Tryptic 'map' of the basic peptides from basic GST enzymes.

The tryptic digests of portions (2.0 mg of protein) of B_1B_1, B_1B_2 and B_2B_2 were separated by high-voltage paper electrophoresis at pH 6.5 in the first dimension and the basic peptides were cut out of the paper as indicated in Fig. 2.2. These peptides were separated in the second dimension by descending chromatography using butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) for 16 h. The stained peptide spots were traced to produce the diagram. No tryptophan-containing peptides were detected. The definition of the symbols is given in Fig. 2.1.

B_1B_2 and B_2B_2 were 43, 47 and 40 respectively of which a total of 36 were common to all three enzymes.

The peptides which were not common to all three enzymes (i.e. the difference peptides), occurred in a manner which is consistent with the hypothesis that transferase B_1B_2 is a heterodimer composed of one subunit which is common to tranferase B_1B_1 and another which is common to transferase B_2B_2. A total of 7 peptides (acidic, neutral and basic) were demonstrated to be common to transferases B_1B_1 and B_1B_2 and not transferase B_2B_2, while 4 peptides were demonstrated to be common to transferases B_2B_2 and B_1B_2. In addition, no peptides were found to be unique to any one of the three enzymes.

3.4c. Estimation of the lysine + arginine content of B_1B_1 and B_2B_2

Tryptic digestion of B_1B_1 and B_2B_2 gave rise to a total of 43 and 40 peptide spots respectively. If it is assumed that B_1B_1 and B_2B_2 are comprised of two identical subunits, then each enzyme should produce 86 and 80 peptides respectively. It follows that the number of tryptic cleavage sites in transferase B_1B_1 would be 85 and in transferase B_2B_2, 79. Since trypsin is specific for peptide bonds associated with the carboxyl groups of lysine and arginine, this suggests that B_1B_1 contains at least 85 arginine and lysine residues per molecule and B_2B_2 79 such molecules. The actual number of lysine + arginine residues/mol, determined by analysis of samples of B_1B_1 and

B_2B_2 following 24 h HCl hydrolysis (Table 4.5) were 76 and 74 respectively. This discrepancy, between observed and predicted values of lysine + arginine content, arises because some tryptic peptides are immobile and remain at the origin during two dimensional peptide mapping (Harris & Hindley, 1965).

3.5 IDENTIFICATION OF THE HYBRID ENZYME GST B_1B_2 AS TRANSFERASE δ

The nomenclature used to describe the basic transferases is based on their elution order from CM-cellulose (Kamisaka et al., 1975).

To enable the hybrid transferase to be identified, a liver of the appropriate phenotype was subjected to the purification scheme of Kamisaka et al. (1975) described in section 2.5a. The liver chosen had previously been shown, using isoelectric focusing, to contain large amounts of B_1B_1 and B_1B_2 protein, but possessed little, if any, B_2B_2 protein or neutral transferase (N_1 or N_2). A portion of extract from this liver was subjected to DEAE-cellulose chromatography followed by CM-cellulose chromatography. The material that was not retained by DEAE-cellulose, was concentrated by ammonium sulphate precipitation before being applied to the CM-cellulose column. Examination of the eluate from this cation-exchanger revealed a broad peak of activity in the flow-through fractions and two major peaks of activity

that were resolved by the salt gradient (Fig. 3.18). The two major peaks of activity resolved by the gradient were identified according to their order of elution, as transferases δ and ϵ respectively.

Using the radioimmunoassays for GST B_1B_1 and B_2B_2, it was found that antisera, raised to transferase B_1B_1, cross-reacted with each of the two peaks of activity eluted by the salt gradient from CM-cellulose. By contrast, the B_2B_2 antisera only cross-reacted appreciably with the first peak on the salt gradient. This peak corresponds to transferase δ described by Kamisaka et al. (1975) (Fig. 3.18). The anti-B_1 and anti-B_2 sera, reacted equally with the transferase δ-containing peak from CM-cellulose, whereas the anti-B_1 serum showed approx. 50-fold greater activity than the anti-B_2 serum for the transferase ϵ-containing peak.

Figure 3.18. Elution profile from CM-cellulose of human liver extract.

The flow-through fractions obtained when human liver extract was applied to a 3.2 cm x 85 cm DEAE-cellulose column were concentrated by $(NH_4)_2SO_4$ precipitation and dialysed against 10 mM potassium phosphate, pH 6.7. The non-diffusible material (0.3 g of protein) was applied to a column (3.2 cm x 56 cm) of CM-cellulose that had been equilibrated with 10 mM potassium phosphate, pH 6.7. After a washing with approx. 600 ml of the running buffer, a 0-150 mM KCl gradient was applied. Fractions (8.6 ml) were collected and the K^+ concentration (■), transferase activity with CDNB (▲) and cross-reactivity with anti-B_1B_1 (●) and anti-B_2B_2 (△) sera were determined.

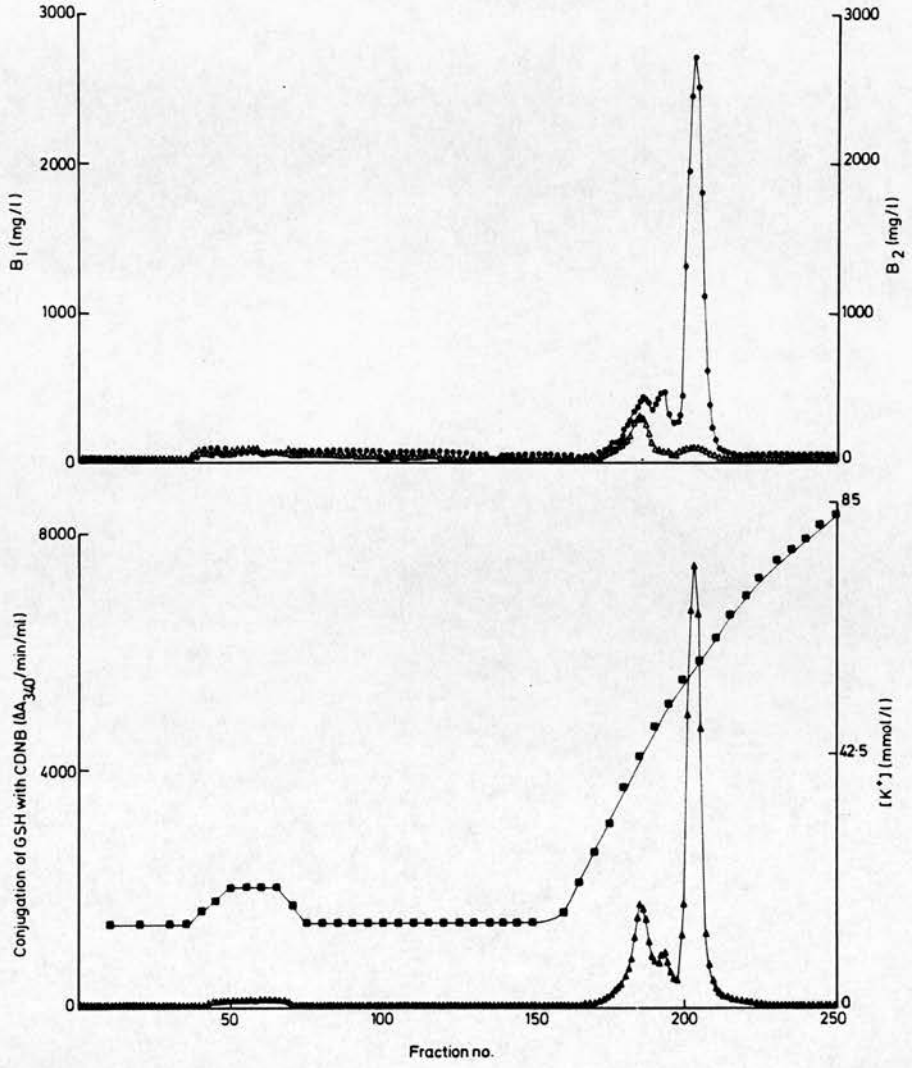

3000
2000
1000
0
B1 (mg/l)
B2 (mg/l)
8000
4000
Conjugation of GSH with CDNB (ΔA340/min/ml)
85
42·5
[K+] (mmol/l)
50
100
150
200
250
Fraction no.

Figure 3.18

SECTION 4: DISCUSSION

4.1 GST CHARACTERIZED DURING THIS PROJECT

a. Current knowledge of rat and human GST enzymes

The glutathione S-transferases are an important group of detoxification enzymes. The cytosolic enzymes are dimeric and in the rat, they are coded for by a limited number of genes. Rat GST comprise hetero- and homodimers, and the large number of forms that have been isolated arise as the result of subunit hybridization (Hayes et al., 1981; Beale et al., 1982, 1983; Mannervik & Jensson, 1982; Hayes, 1983, 1984). Several forms of GST have been purified from human tissues however, their structure and genetic relationship is poorly understood. No hybrid enzyme forms have been described in man and it is widely believed that the human basic transferases represent charge isomers derived from a single gene product.

The aims of this thesis were to: (1) purify the multiple forms of glutathione S-transferase which occur in human liver; (2) determine the catalytic and physicochemical properties of these enzymes; (3) determine the relationship of these enzymes with the glutathione S-transferases which had previously been described; (4) identify if hybrid enzymes exist; (5) define the structural origins of the multiple forms of basic enzyme.

4.1b. Evidence for two families of hepatic GST

The purification of human liver glutathione S-transferases is complicated by the difficulty in

obtaining fresh tissue of a suitable phenotype. Not every individual expresses all forms of the enzyme and a large number of livers were examined to obtain the enzymes studied in this thesis.

Several different purification schemes were used during the study and a total of five hepatic forms of GST were isolated. These have been designated B_1B_1, B_1B_2, B_2B_2, N_1 and N_2. GST N_1 was purified using essentially the method, originally developed in this laboratory, that is based on an initial DEAE-Sephadex step (Hayes et al., 1983; see Table 4.1). Transferases B_1B_1, B_1B_2, B_2B_2, and N_2 were purified by a method that employed DEAE-cellulose rather than DEAE-Sephadex as the first step (Table 4.2).
This latter purification scheme is the most satisfactory of those examined for the purification of all the major forms of hepatic GST (it can also be used to isolate transferase N_1).

A summary of the physicochemical and catalytic properties of the purified human liver GST enzymes is given in Table 4.3. It is clear from these data that the enzymes can be divided into two structurally and functionally distinct groups. The two groups comprise electrophoretically-distinct subunit types, analogous to the rat Ya and Yb subunit types, (see section 4.5), and from a practical point of view are most simply identified by

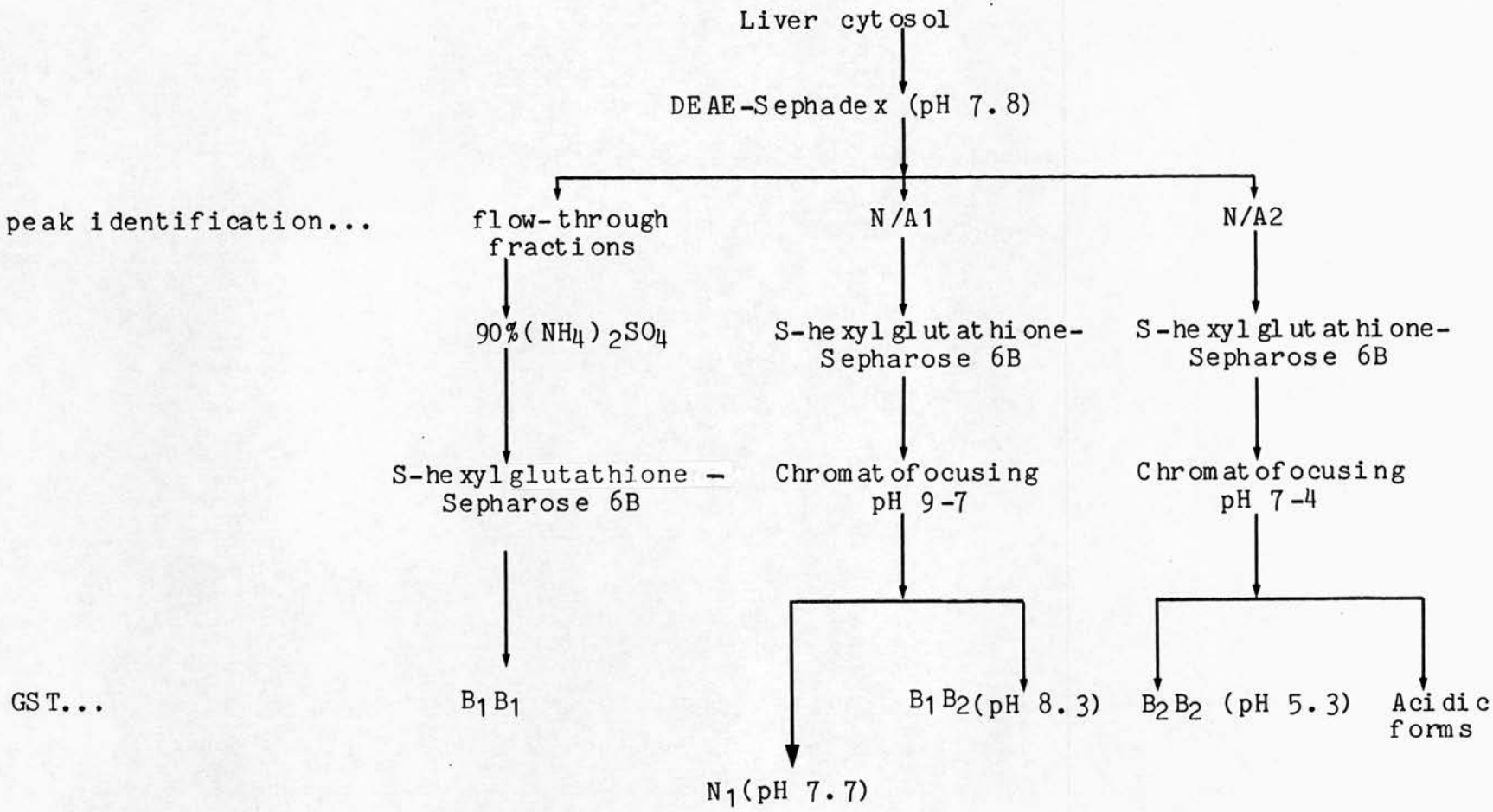


Table 4.1. Purification scheme for GST N_1.

The elution positions of other GST forms are noted for reference.

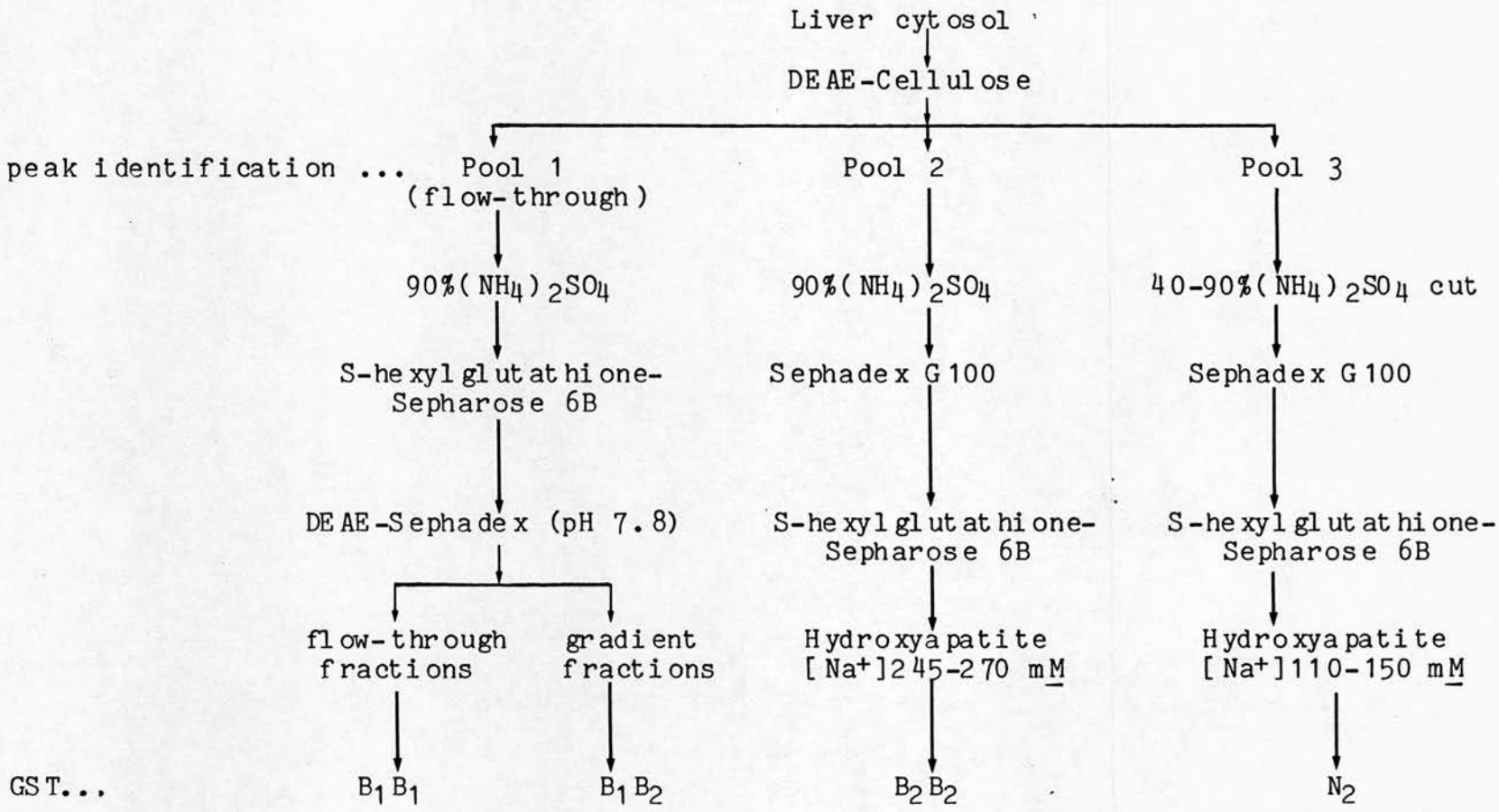


Table 4.2. Purification scheme for basic GST and GST N_2.

their mobility during SDS/polyacrylamide-gel electrophoresis. One group of enzymes (the basic; BB) are characterized by a subunit molecular weight of Mr 25 900, pI >8.0, a high peroxidase activity with cumene hydroperoxide and high sensitivity to the inhibitor tributyltin acetate. The other group (neutral; NN) contains enzymes that are characterized by a subunit molecular weight of Mr 26 500, pI <7, a high activity with trans-4-phenyl-3-buten-2-one and high sensitivity to the inhibitor Cibacron blue.

Enzyme	Subunit Mr	pI value	Characteristic substrate	Characteristic inhibitor
B_1B_1	25 900	8.9	Cumene	Tributyltin
B_1B_2	25 900	8.75	hydroperoxide	acetate
B_2B_2	25 900	8.4		
N_1	26 500	6.1	trans-4-Phenyl-3-buten-2-one	Cibacron* blue
N_2	26 500	4.6		

Table 4.3. Summary of the physicochemical properties of the major forms of GST in human liver. *Result noted by Mannervik (1985).

Table 4.3 shows that all the basic enzymes occur as dimers comprised of subunits of closely similar molecular weight. During the course of this project, eight livers were studied in detail. None of the GST enzymes isolated from these livers was found to be a hybrid form, comprising one subunit from the basic group and one subunit from the neutral group. Therefore, it is unlikely that hybrid

enzymes, consisting of a basic subunit and a neutral subunit can occur as a rare polymorphic event. Further, it should be noted that in the rat, Ya and Yb subunit combinations do not exist, nor can they be formed following reversible denaturation experiments *in vitro* (Boyer *et al.*, 1983). Additional evidence to support the hypothesis that hybrids of basic and neutral GST subunits do not exist, is the fact that the basic and neutral groups of enzymes were found to be immunochemically-distinct (Table 3.6). In the rat, it has been found that the subunits which hybridize display some immunochemical cross-reactivity (Hayes & Mantle, 1986b).

4.1c. Features of the BB group of enzymes

Three forms of human glutathione S-transferase with subunit Mr 25 900 and basic isoelectric points were obtained from each of the livers examined. Reversible dissociation studies (Fig. 3.11) and peptide 'mapping' (Figs. 3.15-3.17) demonstrated that these basic enzymes occur as hetero-and homodimers of two subunits, B_1 and B_2 and have been designated B_1B_1, B_1B_2 and B_2B_2 accordingly. Peptide 'mapping' of the B_1 and B_2 subunits also demonstrated that these subunits differ in their covalent structure.

All three enzymes appear to have identical molecular weights (Fig. 3.7) and very similar amino acid compositions (Table 3.8). They also possess catalytic pro-

perties which are similar. However, marked functional differences can be demonstrated with certain substrates (Table 3.2) and inhibitors (Table 3.3). Such differences will facilitate the identification of the B_1 and B_2 subunits.

Comparisons between the B_1 homodimer and the B_2 homodimer revealed that the B_1 subunit is approximately 2.5-fold more active with the substrate p-nitrophenyl acetate and at least 1000-fold more sensitive to the inhibitory effects of tributyltin acetate (using CDNB as substrate) than the B_2 subunit. The B_2 subunit is approximately 3-fold more active with the substrate 1,2-dichloro-4-nitrobenzene and is at least 10-fold more sensitive to the effects of the inhibitor triethyltin bromide than the B_1 subunit.

Difficulty may be encountered if workers attempt to discriminate between the B_1B_1, B_1B_2 and B_2B_2 enzyme forms on the basis of substrate specificity alone. Table 3.2 demonstrates that at least two of the three basic enzymes have very similar activities with a particular substrate. Indeed, in some cases the activity of the hybrid enzyme is greater than expected from the combined activities of the individual subunits. This phenomenon, which has not been reported to occur in other species, indicates that the catalytic activities of the individual subunits could be inter-dependent. It can be seen from Table 3.3, that an

inhibition assay using tributyltin acetate could be used to discriminate between transferases B_1B_1, B_1B_2 and B_2B_2. However, full identification of the enzymes would be best achieved using a range of substrates and inhibitors.

4.1d. Features of the NN group of enzymes

Two forms of GST with isoelectric points <pH 7 were separately purified from two of the eight livers examined. These neutral-type GST were designated N_1 and N_2 and a summary of their properties is included in Table 4.3. It should be noted that their purification and characterisation was limited by the availability of suitable tissue.

The enzymes N_1 and N_2 are both dimers of subunits of identical molecular weight and are significantly larger than the basic enzymes (Fig. 3.8). They are notable for their high activity with the substrate trans-4-phenyl-3-buten-2-one, and can be separately identified on the basis of their isoelectric points or elution position from chromatofocusing columns (Table 3.1). However, their subunit relationship remains to be established. Analysis of the products of reversible dissociation of GST N_1 suggests that this enzyme is a homodimer. Differences in the substrate specificities of GST N_1 and GST N_2, particularly with ethacrynic, acid suggest that at least two functionally distinct subunits occur in the neutral enzyme group.

4.2 COMPARISON BETWEEN GST DESCRIBED BY DIFFERENT WORKERS

a. Relationship between basic, neutral and acidic GST

In man, the cytosolic GST have been divided into basic, neutral and acidic groups based on their pI values (Warholm et al., 1983). The relationship between the forms that comprise these three groups is not clear. In the literature there is considerable disagreement about many of the physicochemical properties of these enzymes; confusion exists about the immunochemical properties of human glutathione S-transferase, about the subunit composition of individual glutathione S-transferases and about the origin of the polymorphism associated with the hepatic enzymes. For example, the basic and acidic groups of transferases have been conflictingly reported to be either immunochemically inter-related (Awasthi et al., 1980) or, alternatively, immunochemically distinct (Warholm et al., 1983). Furthermore, it has been proposed that all the basic enzymes are heterodimers, composed of a basic subunit and an acidic subunit (Dao et al., 1982; Dao et al., 1984; Singh et al., 1984; Awasthi & Singh, 1985; Singh et al., 1985) or a neutral subunit and an acidic subunit (Hayes et al., 1983). Other workers (Kamisaka et al., 1975; Warholm et al., 1983) have identified basic GST as a homodimer of identical subunits. Several forms of basic glutathione S-transferase have been identified, and the origin of this polymorphism has been attributed to either post-synthetic modification of a

single gene product (Kamisaka et al., 1975; Jakoby & Habig, 1980; Grover, 1982; Strange et al., 1984) or to the presence of distinct allelic genes (Board, 1981a, 1981b). These apparent contradictions between the results from different laboratories, while widely recognised, have prompted surprisingly little analysis by investigators or reviewers (for example, see Mannervik, 1985). Comparisons between the properties of human livers are complicated by the marked phenotypic variation between individuals. Therefore, when the properties of glutathione S-transferases prepared from different livers are not identical, it is difficult to determine if this non-identity is due to methodological variation between laboratories or if it has a genetic origin. The effects of genetic variation can be eliminated by experimenting with portions of liver obtained from a single individual. However, this can be a problem when trying to repeat the work of other investigators, since it is usually not possible to obtain liver samples corresponding to the phenotype originally used.

4.2b. Evidence that GST B_1B_1, B_1B_2 and B_2B_2 are members of the α - ϵ groups of enzymes

Kamisaka et al. (1975) originally described a purification scheme for five basic GST forms (α, β, γ, δ and ϵ) from human liver. These GST forms, differed in isoelectric point (pI 7.8 - 8.8; Table 4.4) but were indistinguishable by other criteria such as amino acid composition (Table 4.5), catalytic activity (Table 4.6),

or immunological identity using Ouchterlony immunodiffusion analysis. These workers proposed that glutathione S-transferases $\alpha - \epsilon$ were coded for by single gene and the multiple forms were generated by a postsynthetic modification, namely, deamidation (Jakoby & Habig, 1980).

The isoelectric focusing data place B_1B_1, B_1B_2 and B_2B_2 in the basic family of glutathione S-transferases. Transferases B_1B_1, B_1B_2 and B_2B_2 exhibit many of the physicochemical properties attributed to the $\alpha - \epsilon$ group of enzymes described by Kamisaka et al. (1975). They have basic isoelectric points and a subunit size (Mr 25 900) equivalent to that of transferases $\alpha - \epsilon$ (Table 4.4). The catalytic properties and amino acid compositions of B_1B_1, B_1B_2 and B_2B_2 suggest that they are members of the same family as transferases $\alpha - \epsilon$.

Transferase	Isoelectric point	Subunit Mr
α	7.8	25 000
β	8.25	25 000
γ	8.55	25 000
δ	8.75	25 000
ϵ	8.8	25 000

Table 4.4. Molecular weight and isoelectric point values of GST $\alpha - \epsilon$. Published for human basic glutathione S-transferase forms α, β, γ, δ and ϵ (Kamisaka et al., 1975).

The amino acid compositions of B_1B_1, B_1B_2 and B_2B_2 are very similar to the amino acid compositions of transferases α, β, γ, δ and ϵ (Kamisaka et al., 1975) and a pooled basic enzyme preparation of Warholm et al. (1983) (Table 4.5). In the latter case, the basic fractions were combined and treated as a single enzyme, thus it is not known if this basic pool contained predominantly B_1 or B_2 subunits. The most significant differences between the amino acid compositions calculated for B_1B_1, B_1B_2 and B_2B_2 and the data presented by the other workers are in the range of recovery of lysine, threonine, serine, alanine, valine and leucine. Nonetheless, these basic transferases can be clearly distinguished from the neutral and acidic forms by this criterion.

As a group, transferases B_1B_1, B_1B_2 and B_2B_2 exhibit catalytic properties which resemble transferases α-ϵ (Table 4.6) and the pooled basic preparation of Warholm et al. (1983) (Tables 4.7 and 4.8). Most notably, GST B_1B_1, B_1B_2 and B_2B_2 express a high peroxidase activity with cumene hydroperoxide and are potently inhibited by tributyltin acetate, both characteristic features of the basic (α-ϵ) group of enzymes (Mannervik, 1985). Values given for the specific activity of transferases B_1B_1, B_1B_2 and B_2B_2 (Table 3.2) are slightly higher than those reported by the other investigators. However, these differences are thought to reflect methodological rather than real

Amino acid	Residues/mol Transferase						Residues/mol Transferase				Residues/mol Transferase		
	α	β1A	β1B	γ	δ	ε	B_1B_1	B_1B_2	B_2B_2	N_1	Basic (α - ε)	Near Neutral (μ)	Acidic (π)
Lys	42	43	42	44	46	44	52	50	50	42	47	40	24
His	6	6	5	5	6	5	7	6	6	10	6	11	4
Arg	23	22	21	22	23	24	24	25	24	19	23	20	16
Asx	34	34	34	33	34	34	39	39	38	45	38	50	42
Thr	6	6	7	7	6	6	10	9	9	13	9	13	18
Ser	12	18	19	17	12	11	26	24	29	20	25	21	20
Glx	54	55	54	54	51	48	52	57	61	26	52	50	49
Pro	22	19	21	19	22	23	23	23	21	21	24	20	24
Gly	20	20	19	21	19	19	28	24	24	22	22	30	37
Ala	25	27	25	29	28	28	35	35	29	19	32	23	32
Val	13	13	13	14	16	15	20	21	18	9	19	13	28
Met	12	14	13	11	12	13	13	15	16	14	15	12	5
Ile	27	29	29	28	28	27	25	30	32	28	30	27	14
Leu	55	58	59	59	58	59	65	65	62	54	58	56	62
Tyr	17	19	18	18	19	18	18	21	20	24	21	24	24
Phe	17	18	18	17	20	18	18	21	20	26	20	26	14

Table 4.5. Amino acid compositions of GST forms obtained by other workers.

The amino acid compositions which have been published for basic forms α, β, γ, δ, and ε (Kamisaka et al., 1975), the pooled basic enzyme preparation (α - ε), GST μ and GST π (Mannervik, 1985). Included for comparison are values for the enzymes purified in this thesis.

Substrate	Specific activity (μmol.min^{-1}.mg^{-1} at 25°C) Transferase				
	α	β	γ	δ	ϵ
1-Chloro-2,4-dinitrobenzene	19	16	17	37	34
1,2-Dichloro-4-nitrobenzene	0.049	0.065	0.035	0.050	0.043
Ethacrynic acid	0.017	0.021	0.035	0.028	0.044
trans-4-Phenyl-3-buten-2-one	0.001	0.001	0.001	0.001	0.002
p-Nitrobenzyl chloride	0.17	0.22	0.019	0.20	0.16
Bromosulphophthalein	0.010	0.010	0.010	0.001	0.004
Iodomethane		1.7		4.2	3.2
Nitroglycerin				0.32	

Table 4.6. Substrate specificities of basic GST forms, α - ϵ .

The substrate specificities which have been published for glutathione S-transferase forms α, β, γ, δ and ϵ (Kamisaka et al., 1975). Note that these were determined at 25°C.

Substrate	Specific activity (μmol.min^{-1}.mg^{-1}) at 25°C Transferases		
	basic ($\alpha - \beta$)	neutral (μ)	acidic (π)
1-Chloro-2,4-dinitrobenzene	64	187	105
1,2-Dichloro-4-nitrobenzene	0.035 - 0.065[a]	0.032	0.11
Bromosulphophthalein	0.001 - 0.01[a]	<0.002	<0.002
Ethacrynic acid	0.017 - 0.044[a]	0.081	0.86
trans-4-Phenyl-3-buten-2-one	0.001 - 0.002[a]	0.36	0.01
1,2-Epoxy-3-(p-nitrophenoxy)-propane	0[a]	0.11	0.37
Styrene-7,8-oxide	0.02	2.6	0.07
Benzo[a]pyrene-4,5-oxide	0.047	0.92	0.13
Cumene hydroperoxide	10.6	0.63	0.03
Δ^5-Androstene-3,17-dione	8.0	0.12	0.01
p-Nitrophenyl acetate	0.18	0.22	0.19

Table 4.7. Substrate specificities of basic, neutral and acidic GST forms reported by other workers.
Substrate specificities of the basic, neutral and acidic groups which have been published in a review on glutathione S-transferases (Mannervik, 1985). The subunit composition of the basic pool was not defined.

[a] Data originally reported by Jakoby & Habig (1980).

Inhibitor	Transferase		
	Basic (α - ϵ)	Near-neutral (μ)	Acidic (π)
Cibacron Blue	5	0.05	0.5
Gossypol acetic acid	50	2	>100
Tributyltin acetate	0.1	0.5	4
Triethyltin bromide	10	5	6
Triphenyltin chloride	0.25	0.5	>10
Bromosulphophthalein	75	2	100
Hematin	0.5	1	5
S-Hexylglutathione	3	10	20
S-(p-Bromobenzyl)glutathione	4	1	4

Table 4.8. Inhibition of basic, neutral and acidic acid GST activity reported by other workers.

IC_{50} values (μm) which have been published for human basic, neutral acidic human glutathione S-transferases. These were determined at pH 6.5, 30°C with 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH as substrates (Mannervik, 1985). The IC_{50} value is the concentration of inhibitor giving 50% inhibition of the enzyme activity.

differences in the proteins; note for example, that the assays were carried out at different temperatures. The general features of transferases B_1B_1, B_1B_2 and B_2B_2, namely, subunit size, isoelectric point, substrate specificity and amino acid composition, indicate that they are closely related to, or part of, the basic family of enzymes described by Kamisaka et al. (1975). By contrast with the results of these workers, who were unable to demonstrate catalytic differences between the individual forms, the results presented in this thesis demonstrate that the individual enzymes are catalytically distinct.

4.2c. Identification of the B_1B_2 heterodimers as GST δ

To establish the relationship of transferases B_1B_1, B_1B_2 and B_2B_2 with the previously described basic enzymes that were designated α - ϵ, each enzyme was subjected to isoelectric focusing and CM-cellulose chromatography; these were the techniques used by Kamisaka et al. (1975) to discriminate between transferases α - ϵ. The B_2B_2 homodimer has a pI of 8.4 and was eluted from the CM-cellulose column at a position equivalent to that originally reported for α, β or γ. The B_1B_1 homodimer has a pI of 8.9 and was eluted from the CM-cellulose column at a position equivalent to ϵ. The B_1B_2 heterodimer possesses the same isoelectric point (pI 8.75) as transferase δ. However, transferase B_1B_2 eluted as two peaks of activity from the CM-cellulose column, both of which cross-reacted

equally with the B_1B_1 and B_2B_2 antisera, and appeared to interconvert when re-applied to the column. Interconverting forms of δ were also described by Kamisaka et al. (1975).

Since these results suggest that transferase δ could be the B_1B_2 heterodimer, transferase δ was prepared by using the original methods of Kamisaka et al. (1975). The CM-cellulose column profile of human basic transferases obtained by the method of Kamisaka et al. (1975; Fig. 3.18) resulted in the clear resolution of transferases δ and ϵ. The cross-reactivity of the fractions with the B_1B_1 and B_2B_2 antisera demonstrated that transferase δ, the first peak of enzyme activity resolved by the KCl gradient, was the hybrid enzyme (B_1B_2). The second major peak of activity resolved by the KCl gradient, transferase ϵ, only cross-reacted with the B_1B_1 antisera, indicating that it was a B_1B_1 homodimer. A minor peak of activity eluted between GST δ and ϵ. This peak, which was not resolved by Kamisaka et al. (1975), only cross-reacted with the B_1B_1 antisera.

Thus, although it is clear that transferases B_1B_2 and B_1B_1 are equivalent to transferases δ and ϵ respectively, it is less clear which basic transferase(s) the B_2B_2 homodimer represents. The isoelectric point of B_2B_2 lies between the values reported by Kamisaka et al. (1975) for transferases β and γ (8.25 and 8.55

respectively). However, in view of the fact that Kamisaka and co-workers could only resolve native cytosol into three peaks of activity by polyacrylamide-gel electrofocusing, it is possible that transferases α, β and γ arise by autoxidation of a single gene product during the purification scheme.

4.2d. GST N_1 and N_2 are members of the neutral group of enzymes

Glutathione S-transferases N_1 and N_2 have a subunit molecular weight which corresponds to that of the neutral transferase μ (Warholm et al., 1983). High specific activity with trans-4-phenyl-3-buten-2-one, previously noted to be characteristic of transferase μ, is also a property of both GST N_1 and N_2. The amino acid composition of transferase μ is virtually identical to that of N_1 with the exception of the recoveries of glycine and glutamic acid/glutamine (Table 4.5). These residues which are significantly higher in the μ preparation may have arisen because glutathione was present in the sample analysed. This source of contamination was present in samples of transferase B_1B_2 prepared for determination of cysteic acid content, despite extensive dialysis against water.

The pI of transferase N_1 (pH 6.1) is within the range reported for transferase μ (pH 6.0 - pH 6.5 Warholm et al., 1981a). However, the isoelectric point of transferase N_2 (pI 4.6) has not been reported for an enzyme of

the neutral-type. This distinctly acidic isoelectric point demonstrates that the designation 'neutral' is inappropriate for this group of enzymes. A re-appraisal of the existing nomenclature is necessary.

4.2e. Are the cationic, neutral and anionic GST inter-related?

Most investigators agree with the evidence presented here that the three groups of human glutathione S-transferases are structurally and immunochemically distinct (Mannervik, 1985). However, Awasthi and co-workers have presented experimental evidence which is at variance with this idea (Awasthi et al., 1980; Dao et al., 1984; Awasthi & Singh, 1985; Singh et al., 1985). They have identified at least eight enzyme forms which arise as dimeric combinations of four immunologically and functionally distinct subunits A, A' B and C (Table 4.9). Cationic, neutral and anionic forms are thought to occur.

Subunit	Subunit Mr	Best Discriminating Substrate
A	26 500	Cumene hydroperoxide
A'	26 500	p-Nitrophenyl acetate p-Nitrobenzyl chloride
B	24 500	Ethacrynic acid trans-4-Phenyl-3-buten-2-one 1-Chloro-2,4-dinitrobenzene
C	22 500	1-2,Dichloro-4-nitrobenzene

Table 4.9. Summary of GST-subunit types reported by Awasthi and co-workers (Singh et al., 1985).

A summary of the proposed subunit combinations and immunological relationships is presented (Table 4.10 and 4.11). The fundamental difference between these results and those from other laboratories is the demonstration that hybrid enzymes occur, comprised by subunits of different molecular weight. Furthermore, antisera raised to the cationic enzymes are reported to cross-react with the neutral and anionic enzymes. Although these structural and immunological relationships have not been reported by other laboratories the apparent contradiction of results has not been discussed.

Awasthi and co-workers examined the subunit structure of purified GST using Urea/SDS/2-mercaptoethanol/polyacrylamide-gel electrophoresis (Singh et al., 1985).

Group	Subunit Structure	pI
Cationic	AB	8.9 8.5 8.3 8.2 8.0
Neutral	BB	6.8
Anionic	A'A' BC	5.5 4.5

Table 4.10. GST subunit combinations, proposed by Awasthi and co-workers.

Subunit structure and isoelectric point of human glutathione S-transferases determined by Singh et al. (1985).

Group	Subunit Structure (Mr)	Cross-reaction between transferase and antiserum: Antiserum: Anti-(AB) cationic	Anti-A subunits	Anti-B subunits	Anti-placental (CC)
Cationic	AB (26 500) (24 500)	+	+	+	-
Neutral	BB (24 500)	+	-	+	-
Anionic	A'A' (26 500)	-	-	-	-
Anionic	BC (24 500) (22 500)	+	-	+	+

Table 4.11. Immunochemical properties of human GST forms, proposed by Awasthi and co-workers.

Immunochemical properties of cationic, neutral and anionic groups of transferases reported by Singh et al. (1985).

Several forms appear to be heterodimers of subunit combinations AB or BC. These dimeric enzymes would be expected to comprise two subunits, for example A and B, which occur in a 1:1 molar ratio and give rise to two equally staining polypeptides. However, the component subunit polypeptides of the cationic enzymes (AB) stained with unequal intensity (Singh et al., 1985). Awasthi and co-workers proposed two possible explanations for this result: either the individual polypeptides differed in their affinity for the dye used to stain the gels, or the cationic enzyme preparation was not homogenous (Singh et al., 1985). If the former explanation is true, then the intensity of staining of the A polypeptide compared to that of the B polypeptide would remain a constant ratio for all samples of the enzyme AB examined. This is not the case, since examination of the original data (Singh et al., 1985) reveals that the intensity of the A polypeptide varies considerably relative to the more predominant B polypeptide. This observation was not noted by the original authors nor did they recognise that the anionic heterodimer (BC) behaves in a similar manner. Thus the electrophoretic behaviour of the cationic and anionic transferases is consistent with these preparations being heterogeneous. It is therefore significant that Awasthi et al. (1980) reported that the cationic and anionic enzymes were homodimers of subunits Mr 24 500 and Mr 22 500 respectively, when they first prepared these enzymes.

The electrophoretic and immunochemical data presented in this thesis do not demonstrate any inter-relationship between the basic, neutral or acidic groups of enzymes.

4.2f GST enzymes that have been isolated as binding proteins

Glutathione S-transferases not only function as enzymes but also as intracellular binding proteins referred to as ligandin (Litwack et al., 1971). A large number of hydrophobic non-substrate molecules bind to GST producing an inhibition of their catalytic activity. This phenomenon has been exploited in the purification of GST and as a means of discriminating between different GST in the rat (Hayes & Mantle, 1986a) and man (Pattinson 1981; Warholm et al., 1983). Furthermore, the differential binding of human transferase forms to a cholic acid affinity matrix (cholic acid-AH-Sepharose 4B) has been exploited to achieve chromatographic separation of the basic enzymes from the acidic form (pI 4.6) present in human liver. The acidic enzyme and possibly neutral forms are retained by this matrix but not the basic enzymes (Pattinson, 1981). Other hydrophobic molecules, such as bilirubin, bromosulphohthalein and deoxycholate, have been shown to bind transferase μ with a much higher affinity than the basic enzymes (α- ϵ) (Warholm et al., 1983; Vander Jagt et al., 1985). These molecules have yet to be used as affinity ligands for the chromatographic separation of transferase μ from basic transferases.

4.2g GST enzymes resolved by GSH-affinity chromatography

Glutathione, a substrate of glutathione S-transferases has also been used as an affinity ligand in the purification of these enzymes. Vander Jagt et al. (1985) have purified human GST using glutathione affinity chromatography, chromatofocusing and hydroxyapatite chromatography (Table 4.12). Two groups of enzymes were identified, based on their interaction with glutathione-Sepharose 6B, the affinity matrix used for the initial purification step. The low-affinity GST forms (L) were eluted from the affinity matrix by merely increasing the pH from 7.4 to 9.6 and the high-affinity GST forms (H) were eluted at pH 9.6 by the inclusion of 5 mM GSH in the buffer.

At least three sizes of subunit were demonstrated to occur within the H and L groups corresponding to those of the basic, neutral and acidic enzymes reported by other workers. Slight differences are apparent in subunit molecular weights reported by Vander Jagt et al. (1985) and the molecular weights reported in this thesis. These are almost certainly due to methodological differences and do not represent novel subunit types. The cross-reactivity data reported by Vander Jagt et al. (1985), appear to support the suggestion that all the basic enzymes (subunit Mr 25 200) are immunologically identical, but distinct from the hepatic neutral glutathione S-transferase

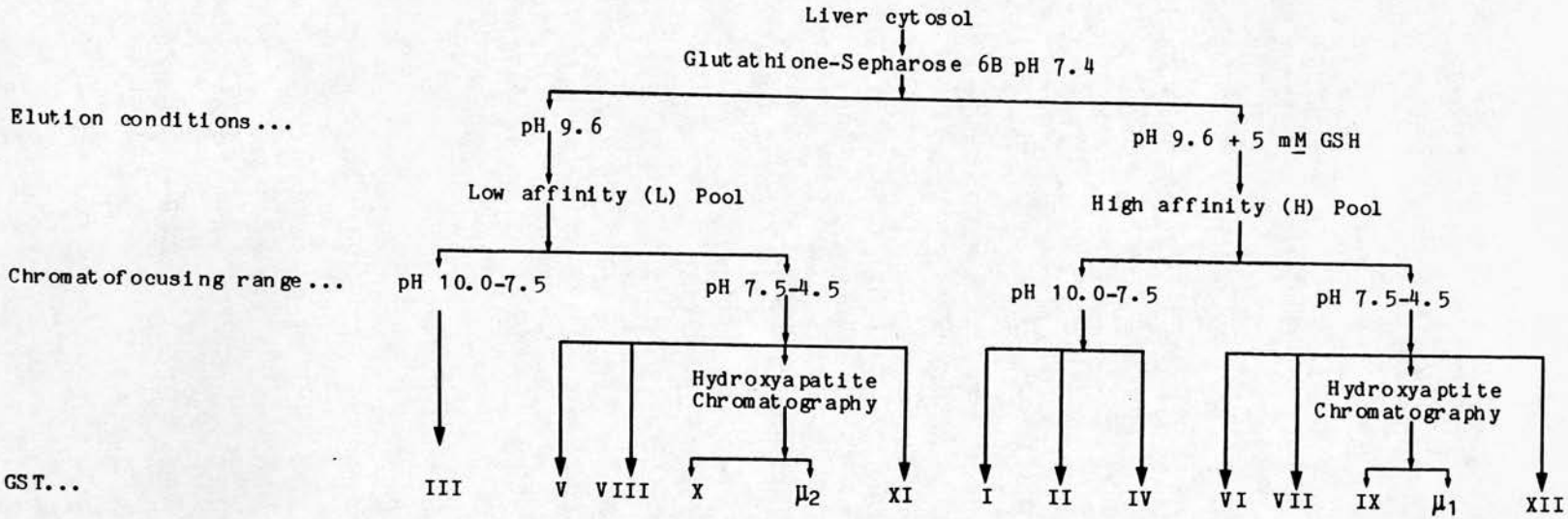


Table 4.12. GSH-affinity chromatography of human GST.

Summary of purification scheme of Vander Jagt et al., 1985.

(subunit Mr 27 900) and acidic glutathione S-transferase forms, erythrocyte (subunit Mr 24 800) and placental (subunit Mr 25 200). However, these workers reported that an acidic type of enzyme, GST XIII (Mr 25 600), present in trace amounts, cross-reacts with the antisera raised against basic glutathione S-transferase.

Fifteen forms of enzyme were purified by this method (Table 4.13). The isoelectric point of each form was determined from the pH of elution from the chromatofocusing column and values over a wide range pH 4.9 - 8.9 were obtained. Isoelectric points of human glutathione S-transferases determined in this way cannot be compared directly with the pI values obtained by other workers using isoelectric focusing (Sluyterman, 1982). The pattern of specific activities of the enzymes with cumene hydroperoxide and 1-chloro-2,4-dinitrobenzene is complex and difficult to interpret in relation to the results of other workers.

Several reports in the literature demonstrate that incubation of GST with glutathione may alter the charge (Ramage & Nimmo 1983) or catalytic properties (Simons & Vander Jagt, 1980; Vander Jagt et al., 1983) of the enzymes. Since the high-affinity group of transferases were eluted with 5 mM GSH, it may be significant that they are much more active with the substrates examined, than the low-affinity enzymes. For example, Simons & Vander Jagt (1980) investigating the inhibitory effects of

bilirubin on human glutathione S-transferase activity, noted that pre-incubation of the basic enzymes with 2.5 mM GSH protected the enzymes from inactivation. In human kidney two basic forms of glutathione S-transferase can be distinguished using an assay based on these observations (Koskelo & Icen, 1984). Glutathione binding is thought to maintain the activity of the kidney enzymes by altering their subunit conformation. The full significance of the effects of ligand binding in transferase activity has yet to be recognised. Bilirubin binding, gossypol binding and protein-protein interactions have all been implicated in the physiological regulation of GST activity (Vander Jagt et al., 1983).

The distinction of the human glutathione S-transferases into high- and low-affinity groups using the affinity chromatography method is arbitrary and may be unrelated to their structural origins. Low affinity forms of enzyme represent only a small proportion of the total protein obtained by Vander Jagt et al. (1985) from the glutathione affinity column. Some of these forms may represent a breakthrough fraction from the high-affinity pool. This breakthrough fraction could account for the large number of enzymes which eluted at equivalent positions when the enzymes from each pool were analysed using chromatofocusing. For example, neutral transferases μ_1 and μ_2 were obtained from the high- and low-affinity pools

respectively, but demonstrated almost identical physicochemical properties; they are most likely to be identical enzyme forms.

A modified version of Table 4.13 is presented in Table 4.14 where the enzyme forms have been re-grouped on the basis of similarities in their reported subunit molecular weight, substrate specificity and apparent pI, in a tentative attempt to relate them to the enzyme groups identified in this thesis. However, these enzymes were purified and analysed by methods distinct from those described for the enzymes in this thesis. Since these techniques are known to alter the catalytic (Vander Jagt et al., 1983) and chromatographic (Ramage & Nimmo, 1983), properties of the enzymes, a direct comparison is therefore not valid. For example, the distinction between μ_1 and μ_2 GST forms may be an artefact. Nonetheless, some similarities in properties do exist which enable the enzymes to be identified in terms of basic, acidic and neutral types.

4.3 IMMUNOCHEMICAL ANALYSIS OF HUMAN GST

a. Discrimination of B_1 and B_2 subunits by radioimmunoassay

The immunological relationship of the basic forms of human glutathione S-transferase ($\alpha - \epsilon$) was first examined by Kamisaka et al. (1975). As a consequence of their work it is widely accepted that these enzymes are immunochemically identical, despite incomplete experimental evidence for this conclusion.

Form	Molecular weight	Affinity for GSH-Sepahrose	Specific Activity $\mu mol.min^{-1}.mg^{-1}$ CDNB	Cumene Hydroperoxide	Apparent pI	Relative amount of GST in liver (%)	Kd (M)
I	25 200	High	68	8.0	8.87	20.4	4×10^{-8}
II	25 200	High	66	6.0	8.74	14.2	8×10^{-8}
III	25 200	Low	62	6.0	8.68	8.1	2×10^{-8}
IV	25 200	High	51	5.0	8.33	5.9	2×10^{-7}
V	25 200	Low	37	2.3	7.42	0.6	10^{-7}
VI	25 200	High	60	5.7	7.35	11.8	3×10^{-7}
VII	25 200	High	68	8.3	7.16	5.1	2×10^{-7}
VIII	25 200	Low	35	2.6	7.09	1.3	9×10^{-8}
IX	25 200	High	35	5.9	6.18	0.5	8×10^{-7}
X	25 200	Low	36	2.7	6.12	0.3	10^{-6}
XI	25 200	Low	33	1.8	5.50	0.6	0
XII	25 200	High	47	8.3	5.32	3.6	4×10^{-5}
XIII	25 600	High	94	2.9	4.82	trace	4×10^{-6}
μ_1	27 900	High	146	1.8	6.18	5.4	10^{-6}
μ_2	27 900	Low	148	2.1	6.12	3.2	5×10^{-7}

Table 4.13. Properties of human liver GST forms I-XIII.

Data originally presented by Vander Jagt et al. (1985) (Liver Sample 1). Dissociation constant (Kd) for the binding of hematin, determined with CDNB as substrate, at pH 6.5 and at 25°C.

Group	Form	Molecular weight	Affinity for GSH-Sepharose	Specific Activity $\mu mol.min^{-1}.mg^{-1}$ CDNB	Cumene Hydroperoxide	Apparent pI	Relative amount of GST in liver (%)	Kd (M)
Basic	I	25 200	High	68	8.0	8.87	20.4	$4x10^{-8}$
	II	25 200	High	66	6.0	8.74	14.2	$8x10^{-8}$
	VII	25 200	High	68	8.3	7.16	5.1	$2x10^{-7}$
Basic	IV	25 200	High	51	5.0	8.33	5.9	$2x10^{-7}$
	VI	25 200	High	60	5.7	7.35	11.8	$3x10^{-7}$
Basic	V	25 200	Low	37	2.3	7.42	0.6	10^{-7}
	VIII	25 200	Low	35	2.6	7.09	1.3	$9x10^{-8}$
	X	25 200	Low	36	2.7	6.12	0.3	10^{-6}
Neutral	μ_1	27 900	High	146	1.8	6.18	5.4	10^{-6}
	μ_2	27 900	Low	148	2.1	6.12	3.2	$5x10^{-7}$
Acidic	XIII	25 600	High	94	2.9	4.82	trace	$4x10^{-6}$
Basic (exception)	IX	25 200	High	35	5.9	6.18	0.5	$8x10^{-7}$
	XII	25 200	High	47	8.3	5.32	3.6	$4x10^{-5}$
Basic (exception)	III	25 200	Low	62	6.0	8.68	8.1	$2x10^{-8}$
	XI	25 200	Low	33	1.8	5.50	0.6	0

Table 4.14. Identification of GST forms I-XIII as members of groups basic, neutral or acidic.

Proposed re-grouping of human liver glutathione S-transferases obtained by Vander Jagt et al. (1985) and originally presented in Table 4.13. This Table is intended as a guide to the identification of GST I-XIII, not as a definitive classification of these forms. Where the properties of enzymes did not permit them to be placed in one particular series, they were designated 'exception'.

The original investigators demonstrated that each of the basic enzyme forms (α - ϵ) cross-reacted with anti-ligandin antiserum. However, the purity, subunit composition and method of preparation of the ligandin preparation that was used as immunogen was not described. This prevents their cross-reactivity data from being interpreted at a subunit level.

Using a radioimmunoassay, Hayes and co-workers were able to distinguish between two Ya-type subunits in human liver (Hayes et al., 1983; Beckett & Hayes, 1984); the GST enzymes comprising these Ya subunits are now known to be basic enzymes. These workers purified several enzymes, that were originally designated Basic, N/A1 and N/A2b. The immunochemical cross-reactivity data (Beckett et al., 1985; Table 4.15) indicated that the GST forms, designated Basic and N/A2b, were distinct homodimers. The relationship between GST B_1B_1, B_1B_2 and B_2B_2 and transferases N/A1, N/A2b and Basic was examined using the antisera and original protein standards prepared by Hayes et al. (1983). Cross-reactivities, determined using the radioimmunoassay procedure are given in Table 4.16, and demonstrate that transferases Basic, N/A1 and N/A2b correspond to transferases B_1B_1, B_1B_2 and B_2B_2 respectively. The antisera and protein standards have been re-named accordingly.

Transferase	Relative cross-reactivity (%)	
	Anti-N/A2b GST	Anti-Basic GST
Basic	0.6	100
N/A_1	58	55
N/A2b	100	0.2
Acidic (Erythrocyte; ρ)	<0.1	<0.1

Table 4.15. Specificities of antisera used in the radioimmunoassay of GST N/A2b and GST Basic.

Data originally published by Beckett et al., 1985. The relative cross-reactivity was defined as that quantity of GST which was required to produce 50% displacement of bound ligand when compared with the most immunoreactive GST.

Transferase	Relative cross-reactivity (%)	
	Anti-N/A2b GST	Anti-Basic GST
B_1B_1	<0.2	100
B_1B_2	50	40
B_2B_2	100	<0.2

Table 4.16. Immunochemical relationship between GST B_1B_1, B_1B_2, B_2B_2 and antisera raised to GST N/A2b and GST Basic.

Preparation of GST N/A2b and GST Basic described by Hayes et al., (1983).

4.3b Comparison between the analysis of GST by radioimmunoassay and 'Western' blotting

It is apparent that the 'Western' blot analysis is less specific than the radioimmunoassay using the same sets of antisera. The anti-B_1B_1 and anti-B_2B_2 antisera cross-react with basic forms of enzyme but not neutral or acidic forms using either 'Western' blot analysis or radioimmunoassay. However, by contrast to radioimmunoassay, 'Western' blot analysis does not discriminate between the B_1 and B_2 subunits. This difference in the cross-reactivity, observed in these two types of assay, may be related to the dilution at which the antisera are used. The concentration of antiserum required to detect the corresponding immunogen using 'Western' blot analysis is approximately 10 times higher than that required for the radioimmunoassay. As a consequence of this, the binding characteristics of the antisera are not identical. High-affinity antibodies, specific to the immunogen, contribute predominantly to the binding detected using the radioimmunoassay. However in addition to these, sub-populations of low-affinity antibodies, not specific to the immunogen, contribute significantly to the binding detected in 'Western' blot analysis.

Despite the fact that the' Western' blotting method does not distinguish between individual basic forms, it is a valuable method for unambiguously distinguishing basic from neutral and acidic glutathione S-transferase.

4.4 GENETIC BASIS FOR MULTIPLE GST

a. Zymogram analysis

Mannervik (1985) considers that the marked physico-chemical differences between the basic, neutral and acidic groups of enzymes have a genetic origin and that these enzymes are the products of three gene loci. It is, therefore, significant that when Board (1981a, 1981b) analysed the expression of human GST in the population he separated these enzymes into three sets. From an analysis of zymogram patterns of human liver cytosols he described the existence of three electrophoretically distinct groups of GST. Although not specifically stated, the patterns observed were consistent with the existence of two basic subunits, two neutral subunits and one acidic subunit. Hybrid combinations of each type of basic or neutral subunit could be predicted from this analysis.

Board (1981a) demonstrated that when liver cytosol was subjected to starch-gel electrophoresis at pH 8.6, multiple bands of glutathione S-transferase activity were revealed, with 1-chloro-2,4-dinitrobenzene as substrate, and these were divided into three sets. A strongly staining set which migrated towards the anode were proposed to be the products of one polymorphic gene locus, GST 1. Enzymes assumed to be the products of this locus were described by one of four possible electrophoretic patterns: a fast anodal band, a slow anodal band, a three-banded pattern and an absence of detectable acti-

vity. These are consistent with the expression of three possible alleles GST 1*0, GST 1*1 and GST 1*2 which can occur in the proposed phenotypes GST 1 1, GST 1 2, GST 1 2-1 and GST 1 0.

The set of enzymes that migrated towards the cathode were considered to be products of the GST 2 locus and gave rise to one of three electrophoretic patterns characterteristic of a polymorphic gene locus: a fast cathodal band, a slow cathodal band or a three-banded pattern. These patterns are consistent with the expression of two possible alleles GST 2*1 and GST 2*2 which can occur in the phenotypes GST 2 1, GST 2 2 and GST 2 1-2.

A third set of weakly staining enzymes with fast anodal mobility were considered to be the products of the GST 3 locus. Board (1981a) observed a single band at this locus indicative of a single allele (GST 3*1). Thus, Board postulated that these multiple bands detected on starch-gel electrophoresis represented allelic variants of human glutathione S-transferase. The triplet patterns exhibited by the GST 1 and GST 2 loci are characteristic of those obtained from heterozygotes for a polymorphic dimeric protein. He demonstrated that each of the alleles appeared to be in Hardy-Weinberg equilibrium, since the phenotypes observed corresponded closely with the theoretical distribution.

This proposal, namely that two alleles were present at the GST 2 locus, one allele at the GST 3 locus and three alleles at the GST 1 locus can be applied to the polymorphism of the glutathione S-transferases described in this thesis.

The charge and frequency of occurrence of the neutral (NN) group of enzymes is consistent with them representing the products of the GST 1 locus. Only two of the eight livers examined in this thesis expressed distinct neutral forms, however, their phenotype has yet to be established. This result is surprising since other workers have demonstrated that approximately 60% of the population expressed the neutral enzyme (μ) previously thought to be the only neutral form (Warholm et al., 1983). A small sample population may have given rise to the low frequency of the neutral enzymes obtained in this study.

The basic group of enzymes (BB) have been formally identified to be dimeric combinations of two subunits B_1 and B_2. The charge, frequency of occurrence and multiple band patterns of the GST 2 locus is consistent with it representing the basic (BB) group of enzymes. Subunits B_1 and B_2 appear to be the allelic products of this locus.

Minor acidic forms of human glutathione S-transferase were identified in several livers but not fully investigated, because of lack of material. These acidic

forms are thought to represent the weakly staining products of GST 3.

4.4b Post-synthetic modification of GST

An alternative interpretation of the multiple band patterns at the GST 2 and GST 3 loci originally described by Board, (1981a, 1981b) has been proposed by Strange et al. (1984). While these workers agree that the human glutathione S-transferases are the products of three gene loci they consider that multiple bands can be generated by post-synthetic modification of a single gene product. Strange et al. (1984) noted that the patterns of multiple bands at the GST 2 and GST 3 loci were not a constant individual characteristic but were related to tissue type. For example, a human adrenal cytosol was reported to exhibit a single predominant, most cathodal, GST 2 band. However, the liver cytosol from the same individual was reported to give rise to three bands at this locus. Strange et al. (1984) consider that the strong cathodal GST 2 enzyme that is present in adrenal cytosol represents the parental protein from which other GST 2 forms arise post-synthetically. However, the observation that the band patterns are not identical between tissues of the same individual does not in itself demonstrate that post-synthetic modification of glutathione S-transferase occurs. Indeed, these workers were unable to demonstrate modification of human glutathione S-transferase forms

which were incubated *in vitro* with sulphydryl reagents or stored at 4°C for up to 96 h.

The origin of the differences in band patterns between organs of the same individual may have a genetic basis. For example, in rat liver, the genes for the Ya, Yc and Yb subunits appear to be separately controlled by transcriptional activation (Ding & Pickett, 1985). This can lead to differences in accumulation of the separate gene products and may determine the pattern of GST expression in other organs.

Undoubtedly, the full extent of variation of human glutathione S-transferases has yet to be established. The sensitivity of the zymogram technique only permits identification of major enzyme forms and more sensitive high-resolution techniques, such as isoelectric focusing in polyacrylamide-gels followed by 'Western' blotting, may undoubtedly reveal more forms.

4.5 RELATIONSHIPS BETWEEN GST FROM MAN AND THOSE FROM OTHER MAMMALIAN SPECIES

Glutathione S-transferases are widely distributed in the animal kingdom suggesting that they play an important biological role. In virtually all species studied to date, these enzymes have been found to occur as multiple forms. However, the reasons for the existence of multiple forms and their evolutionary origin is poorly understood. Comparisons between the glutathione S-transferases from different species have been complicated

because of the confusing series of nomenclatures used to identify these enzymes. A survey of the properties of the major isoenzyme families in several mammalian species has been carried out (Hayes & Mantle, 1986c; Mannervik et al., 1985) to help to establish if they have a close phylogenetic relationship. The cytosolic glutathione S-transferases from rat, mouse and man have been shown to share structural, catalytic and immunological properties.

The grouping of glutathione S-transferases with similar properties can be used for a species independent classification. Enzymes from each species can be subdivided into three groups, comprising isoenzymes with common characteristic properties. It is suggested that these groups of enzymes are the products of three distinct gene loci. Mannervik et al. (1985) have called these the alpha, mu and pi groups and these correspond to the basic, neutral and acidic groups respectively, in man.

Functional homology is apparent between glutathione S-transferases of the same groups obtained from different species (Mannervik et al., 1985). For example, basic (BB) enzymes from man, YaYa and YcYc enzymes from the rat and mouse transferase MI, exhibit high 3-ketosteroid isomerase activity and glutathione peroxidase activity. Similarly neutral (NN) transferases in man, rat Yb_1Yb_1 and Yb_2Yb_2 transferases and mouse MIII transferase are active with substrates trans-4-phenyl-3-buten-2-one as well as p-nitrobenzyl chloride. Human acidic transferase (π),

rat transferase YfYf and mouse transferase MII are all active with ethacrynic acid as substrate. The effects of specific inhibitors follow a similar pattern for the enzymes from different species. Basic human transferases (BB) and the equivalent rat (YaYa and YcYc) and mouse (MI) forms have low IC_{50} values for hematin and high values for Cibacron blue; neutral (NN) human transferases and equivalent rat (Yb_1Yb_1 and Yb_2Yb_2) and mouse (MIII) forms have a high IC_{50} group for hematin and a low value for Cibacron blue. A low IC_{50} value for triphenyltin chloride distinguishes the neutral (NN) type enzymes from the acidic (π-like) forms.

The cytosolic enzymes in every species examined are dimers and the subunits have similar sizes (Table 4.17). During SDS/polyacrylamide-gel electrophoresis, the basic (BB) glutathione S-transferases have a mobility similar to the rat Ya subunit, the neutral (NN) enzymes migrate with the rat Yb subunit and the acidic enzyme (λ) migrates with the rat Yf subunit (Hayes & Mantle, 1986c). 'Western' blot analysis of rat and human enzymes (Table 4.17) has shown that the human basic (BB) glutathione S-transferase subunits are immunochemically related to the rat Ya subunit. Human neutral glutathione S-transferase subunits (NN) are related to the rat Yb_1, Yb_2 and Yn transferase family and human acidic subunits (λ) are immunochemically related to rat Yf glutathione S-transferase

subunits. This immunoblot approach has been used to show that the hepatic glutathione S-transferases in the mouse comprise Yf, Ya and Yb subunits, those in hamster comprise Ya, Yb and Yc subunits, and those in guinea pig comprise Ya and Yb subunits (Hayes & Mantle, 1986c).

It is evident from the N-terminal amino acid sequences of human, rat and mouse transferases (Table 4.18) that the enzymes from the same group, but different species, are more closely related than those enzymes of different groups from the same species. Nucleotide sequences of cloned cDNA for rat subunits have revealed that there is approximately 25% homology between subunits from different groups and at least 66% homology between subunits within a group (Ya/Yc) (Ding et al., 1985). Mannervik et al. (1985) postulated that the three groups of enzymes alpha, mu and pi arose by gene duplication before the evolution of different mammalian species. The inclusion of the Yc subunit in the alpha group of enzymes as suggested by Mannervik et al. (1985) may not be valid (Li et al., 1986). This subunit is expressed in two species, rat and hamster but has yet to be detected in other species studied, including man (Hayes & Mantle, 1986c; Tu & Qian, 1986, Tu et al., 1986). A classification of the human subunit types identified in this thesis, based on their electrophoretic mobility, is given in Table 4.19.

Molecular weight (Mr) of immunoreactive polypeptides with antisera raised against rat glutathione S-transferases

		Rat enzymes				Human enzymes	
Species	Tissue	Anti-YaYa	Anti-Yb_1Yb_1	Anti-YcYc	Anti-YfYf	Anti-B_1B_1	Anti-λ
Rat	Liver	25 500	26 300	27 500	N.D.+	(25 500)	N.D.*
Man	Liver	(25 900)	26 500	N.D.	N.D.+	25 900	N.D.
Mouse	Liver	(25 800)	26 400	N.D.	24 800	(25 800)	24 800
Hamster	Liver	26 000	26 700	27 000	N.D.	26 000	N.D.
Guinea pig	Liver	25 300	26 000	N.D.	N.D.	25 300	N.D.

Table 4.17. Immunological relationships between GST subunits from different species.

Apparent sizes of Ya, Yb, Yc and Yf type subunits in various species obtained from Hayes & Mantle, (1986c). Electrophoresis was performed using 12% (W/V) polyacrylamide gels that contained 2.6% (w/v) N,N'-methylenebisacrylamide. 'Western' blotting was carried out as described in this thesis. Values in parenthesis represent weakly positive results. Abbreviation N.D., not detected.

*Anti-λ antiserum cross-reacts with a polypeptide (Mr 24 800) in rat lung.

+Anti-YfYf " " " " " (Mr 24 800) " " "

Group	Transferase	Species	Amino acid Sequence
alpha	YaYa* (cDNA)	Rat	(M)SGKPVLHYFNARGRMECIRWLLAAA
alpha	YaYc*	Rat	PGKPVLHYFNGRGRMEPI
alpha	YcYc (cDNA)	Rat	(M)PGKPVLHYFDGRGRMEPI
alpha	YcYc	Rat	PGKPVLHYF
alpha	(α- ϵ)	Man	S K ? Blocked N-terminus
alpha	MI (YaYa)	Mouse	Blocked N-terminus
mu	μ	Man	PMILGYWDIRGLAHAIRLLLEYT
mu	Yb_1Yb_1 (cDNA)	Rat	(M)PMILGYWNVRGLTHPIRLLLEYT
mu	Yb_1Yb_1	Rat	PMILGYWNVRGLTHPIRLL
mu	Yb_2Yb_2 (cDNA)	Rat	(M)PMTLGYWDIRGLAHAIRLFLEYTDT
mu	Yb_2Yb_2	Rat	PMTLGYWDIRGLAHAIRLFLEYTDT
mu	MIII (YbYb)	Mouse	PMILGYWNVRGLTHPIRMLLQYT
mu	GT-8.7	Mouse	PMILGYXNVRGLXHPIRMLLEYXDX
mu	GT-9.3	Mouse	PMTLGYWNTRGLTHSIRLLLEYXDS
mu	Bovine	Cow	PMILGYWDIRGLAHAISLLL
pi	Erythrocyte (ρ)	Human	PPYTVVYFPVRGRCAALRMLLAD
pi	Lung (λ)	Human	PPYTVVYFPVRGRCAALRMLLAD
pi	Placenta (π)	Human	PPYTVVYFPVRGRCAALRMLLAD
pi	YfYf (cDNA)	Rat	(M)PPYTIVYFPVRGRCGATRMLLAD
pi	YfYf	Rat	PPYTIVYFPV
pi	MII (YfYf)	Mouse	PPYTIVYFPVVDGCEAM

Table 4.18. Amino-terminal amino acid sequences of GST enzymes.
Residues within parenthesis indicate initiator methionine.
cDNA, refers to amino acid sequences determined from the cloned cDNA nucleotide sequence.
X, refers to residues which have not been determined.
*Ya amino-terminal is blocked.

References to Table 4.18

Group alpha	YaYa (cDNA)	Pickett et al. (1984)
	YaYa	Lai et al. (1984)
	YaYa	Taylor et al. (1984)
	YcYc (cDNA)	Telakowski-Hopkins et al. (1985)
	YaYc	Frey et al. (1983)
	YcYc	Mannervik et al. (1985)
	(α - ϵ)	Alin et al. (1985b)
	MI	Mannervik et al. (1985)
Group mu	μ	Alin et al. (1985b)
	Yb_1Yb_1 (cDNA)	Ding et al. (1985)
	Yb_1Yb_1	Mannervik et al. (1985)
	Yb_2Yb_2 (cDNA)	Ding et al. (1986)
	Yb_2Yb_2	Mannervik et al. (1985)
	MIII	Mannervik et al. (1985)
	GT-8.7	Pearson et al. (1983)
	GT-9.3	Pearson et al. (1983)
	Bovine	Asaoka (1984)
Group pi	Erythrocyte (ρ)	Alin et al. (1985b)
	Lung (λ)	Dao et al. (1984)
	Placenta (π)	Dao et al. (1984)
	MII (YfYf)	Mannervik et al. (1985)
	YfYf (cDNA)	Sugioka et al. (1985)
	YfYf	Mannervik et al. (1985)

Species	Subunit Type	Subunit Size (Mr)	Group
Rat	Yc	27 500	alpha
Rat	Ya	25 500	alpha
Man	B_1	25 900	alpha
Man	B_2	25 900	alpha
Mouse	Ya (MI)	25 800	alpha
Rat	Yb_1	26 300	mu
Rat	Yb_2	26 300	mu
Man	N_1	26 500	mu
Man	N_2	26 500	mu
Mouse	Yb (MIII)	26 400	mu
Rat	Yf	24 800	pi
Man	λ	24 800	pi
Man	ρ	24 800	pi
Mouse	Yf (MII)	24 800	pi

Table 4.19. Classification of the human GST subunits.

Based on subunit molecular weight data from this thesis and immunochemical data from Hayes & Mantle (1986c).

4.6 PHYSIOLOGICAL ROLE OF GST

a. Evidence for GST involvement in drug metabolism

While the physico-chemical and catalytic properties of many forms of glutathione S-transferase have been established, it has proven difficult to relate these to the activities of the species or organ from which they came.

The multiplicity of glutathione S-transferase forms and their overlapping substrate specificities have led a number of workers to suggest that a major role of these enzymes is to protect cells from mutagens and carcinogens (Jakoby & Habig, 1980). Evidence in support of this theory has been obtained from studies of glutathione S-transferase expression in plant and animal tissues which have become resistant to the action of cytotoxic chemicals (Summary, Table 4.20).

Cells, when exposed to cytotoxic compounds have the capacity to become resistant by reducing the intracellular concentration of these toxins and also by limiting the damage which they produce. Induction of detoxification enzymes is an important mechanism in producing the drug resistant phenotype and examples of this process with respect to glutathione S-transferase have been documented. Often exposure to a single agent results in the development of cross-resistance to other drugs with markedly different structures and mechanisms of action (Wolf et al., 1987). The development of multidrug resistance is usually accompanied by a decrease in net cellular accumulation of

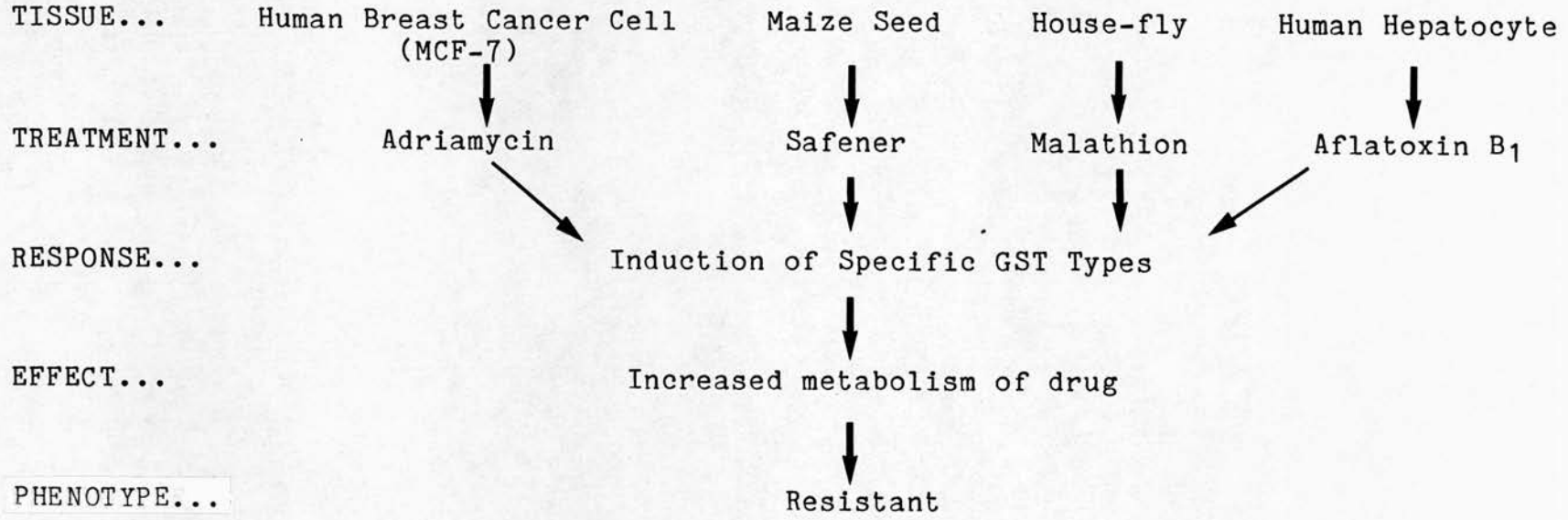


Table 4.20. Model systems for GST-induced drug resistance.

Summary of the biological models in which induction of glutathione S-transferases has been associated with resistance to the effects of cytotoxic drugs. Further details are available in the text. It must be emphasised that several enzyme detoxication systems are induced in these tissues in addition to GST and undoubtedly play a role in the development of multidrug resistance.

Notes to Table.

Adriamycin is an anti-tumour antibiotic.
Safeners are chemical antidotes which selectively protect maize from injury by chloroacetanilide herbicides.
Malathion is an organophosphorous insecticide.
Aflatoxin B_1 is a fungal product which is carcinogenic. Aflatoxin is not itself an inducing agent.

a drug and an increase in specific membrane transport proteins or detoxication enzymes. Elevation of specific glutathione S-transferase isoenzymes has been observed in four model systems of multidrug resistance under investigation: (1) human tumour cells in response to adriamycin treatment (Batist et al., 1986); (2) maize seedlings in response to herbicide treatment (Wiegand et al., 1986); (3) insects in response to organophosphorous insecticides (Motoyama et al., 1980); (4) precancerous, or pre-neoplastic, rodent and human cells (Farber, 1984).

4.6b GST and resistance of tumour cells to anti-cancer antibiotics

Batist and co-workers (Batist et al., 1986) selected a human breast cancer cell line (Adr^R MCF-7) which was made resistant to the anti-tumour antibiotic adriamycin in vitro. These workers found that this cell line exhibited cross-resistance to a wide range of anti-cancer drugs (Table 4.21). The multidrug resistant phenotype was found to be associated with increases in glutathione S-transferase activity. A 45-fold increase in glutathione S-transferase activity was due to the expression of an acidic (pI 4.8) glutathione S-transferase isoenzyme in the Adr^R MCF-7 cells. This enzyme was found to be immunochemically similar but catalytically distinct from the acidic glutathione S-transferases (λ, π, ρ) described in this thesis. While the size and charge of the enzyme were similar

to those described for GST λ (from human lung) and GST ρ (from human red cell) it demonstrated a high substrate specificity for cumene hydroperoxide (normally associated with basic GST) and ethacrynic acid (normally diagnostic for acidic enzyme forms).

Drug	WT	Adr^R MCF-7	-Fold resistant
	IC_{50} (μM)		
Adriamycin	0.025	4.8	192
Actinomycin D	0.002	0.72	357
Vinblastin	0.0014	0.38	274
VP-16	0.4	70	175
Vincristin	4.1	>700	>170

Table 4.21. Sensitivity of human cell lines to anti-neoplastic chemicals.

Wild type (WT) and adriamycin resistant (Adr^R MCF-7) cells.
Reproduced from Batist et al. (1986). The results are expressed as IC_{50}.

IC_{50}, refers to the concentration of anti-neoplastic drug required to kill 50% of the cells.

4.6c GST and resistance of plant cells to herbicide treatment

Treatment of maize seeds with chemical antidotes increases the tolerance of the resulting maize plants to herbicides. These chemical antidotes, for example, N,N-diallyl-2,2-dichloroacetamide, also referred to as safeners, induce resistance which is associated with a 2-3 -fold increase in GST activity (Wiegand et al., 1986).

Maize contains a family of GST enzymes which are believed to be involved in various aspects of glutathione metabolism as well as detoxification of certain herbicides. These enzymes are dimeric, and three forms designated GST I, GST II and GST III have been obtained from this tissue. GST I is a homodimer of two subunits of Mr 29 000 and is expressed constitutively in maize. On treatment with safener there is a two-fold increase in GST I. A novel form, GST II, that is not found in untreated maize is also induced. GST II has a high specific activity with the herbicides alachlor and atrazine and is a heterodimer composed of one subunit Mr 29 000 identical to that found in GST I and an additional subunit (Mr 27 000) not expressed in untreated tissue. Both GST I and GST II can catalyse the formation of glutathione -herbicide conjugates in vivo.

4.6d GST and resistance of pre-cancerous cells to alkylating agents

Resistance to anti-cancer drugs is a feature of early or pre-cancerous stages of cancer development and is associated with adaptive changes in certain detoxication enzymes including glutathione S-transferase. These are thought to reflect an adaptation by the cells, conferring resistance to the cytotoxic effects of the carcinogens to which they are exposed. However, this response also results in an enhanced capacity to inactivate anti-cancer agents, making the tumour resistant to chemotherapy.

Exposure of cells to radiation or cytotoxic chemicals leads to new and altered pre-neoplastic cell populations. These cells may revert to a normal cell type but have the potential to develop to the cancerous state. The biological and biochemical aspects of this process have been extensively studied by Farber and his colleagues (Farber, 1984).

Chemically-induced carcinogenesis occurs in stages, each of which has increased the probability of progression to a malignant state. The pre-cancerous state in humans can last 30 years before autonomous growth, invasion and metastasis occur. Profound biochemical and physical changes develop very rapidly after cells have been exposed to carcinogens, often within hours. In human liver, the pre-cancerous tissue exhibits a decrease (75-90%) in total microsomal cytochromes, P-450 and b_5. However, a large

increase (2-15-fold) in several detoxication enzymes (glutathione S-transferases, UDP-glucuronyltransferase, DT-diaphorase, microsomal epoxide hydrolase and γ-glutamyltransferase) occurs. A significant change in gene expression accompanying pre-neoplasia is that of the GST Yf subunit. This subunit has also been referred to as P21 (Eriksson et al., 1983) and Yp (Kitahara et al., 1984; Satoh et al., 1985). GST Yf is not found in normal rat liver and it has been estimated using 'Northern' and 'Western' blot analysis that this subunit is induced about 100-fold in experimental hepatocarcinogenesis (Sugioka et al., 1985).

Induction of Yf in the rat gives rise to a homodimer, YfYf, which accounts for 85% of the cytosolic activity measured with 1-chloro-2,4-dinitrobenzene. It has a neutral isoelectric point (pI 6.9) and is active with ethacrynic acid as substrate. Since YfYf is not expressed in normal rat liver, it acts as a marker for tumour development in this tissue. However, other rat tissues, including lung, kidney, placenta and heart, express YfYf (Jensson et al., 1985; Kodate et al., 1986; Pemble et al., 1986; Soma et al., 1986; Moore et al., 1987).

4.6e Role of GST in the development of resistance to cytotoxic chemicals

The observation, that glutathione S-transferase isoenzymes are induced in cells selected for multi-drug

resistance and in human hyperplastic nodules resistant to various cytotoxic chemicals, is very important in our understanding of the *in vivo* functions of these enzymes. For the first time, a link between the catalytic properties of glutathione S-transferase and the protection of cells from the effects of cytotoxic compounds, has been described.

Development of resistance to the anti-tumour antibiotic adriamycin, appears to be due to an increase in cellular peroxidase activity associated with induced glutathione S-transferase isoenzymes (Batist *et al.*, 1986). Many of the cytotoxic effects of adriamycin are thought to be caused by the production of superoxide radicals (O_2^-) following microsomal activation of the drug to form reactive semiquinones. Glutathione S-transferase-mediated peroxidase activity can reduce these highly reactive organic peroxides to less reactive alcohols. A 2-3-fold reduction in intracellular drug accumulation, concomitant with a marked decrease in the formation of hydroxyl radicals, occurs in the resistant cell line, Adr^R MCF-7, following exposure to adriamycin (Batist *et al.*, 1986).

Induced peroxidase activity may have a role in the development of cross-resistance to other anti-neoplastic agents which act by a similar mechanism to adriamycin. For example, actinomycin D toxicity is thought to be caused by an increased production of oxygen radicals.

Interestingly, adriamycin-resistant cells (Adr^R MCF-7) have a greater resistance to actinomycin D than to the selecting agent (Table 4.21). The induction of cross-resistance to anti-neoplastic agents which act by a mechanism distinct from that of adriamycin (e.g. vincristine and vinblastine, interfere with microtubule formation) has yet to be explained. It is most likely that these drugs are eliminated by detoxification enzymes which are separate from the glutathione S-transferases. Alternatively, they may be subject to a glutathione S-transferase catalysed reaction which has yet to be identified.

4.7 CONCLUDING REMARKS

The central objective of this thesis was to establish the molecular relationship between the GST forms in human liver. The data obtained led to the suggestion that the basic enzymes in man comprise two distinct subunits, designated B_1 and B_2. Recently, confirmation of this hypothesis has come from sequence analysis of cDNA encoding the basic human GST. Rhoads *et al.* (1987) have characterized two distinct cDNA sequences which are 95% homologous; these sequences are referred to as pGTH1 and pGTH2 and probably correspond to B_1 and B_2 respectively. Similarly, from an analysis of human GST forms, Soma *et al.* (1986), Oustlund Farrants *et al.* (1987) and Tateoka *et al.* (1987) have identified two basic subunits.

The isolation of two distinct forms of neutral GST (N_1 and N_2), demonstrates that this group of enzymes is more complex than previously thought.

None of the data presented in the thesis suggest that the basic, neutral or acidic forms of GST are structurally inter-related.

SECTION 5: REFERENCES

Agrup, G., Arup, P., Andersson, T., Falck, B., Hansson, J-A., Jacobsson, S., Rorsman, H., Rosengren, A.M. and Rosengren, E. (1975) Urinary excretion of 5-S-cysteinyl-dopa in patients with primary melanoma or melanoma metastasis. Acta Dermatovener., (Stockholm) 55: 337-341.

Agrup, G., Falck, B., Rorsman, H., Rosengren, A.-M. and Rosengren, E. (1977) Glutathionedopa in malignant melanoma. Acta Dermatovener., (Stockholm) 57: 221-222.

Alin, P., Danielson, H. and Mannervik, B. (1985a) 4-hydroxyalk-2-enals are substrates for glutathione transferase. FEBS Lett., 179: 267-270.

Alin, P., Mannervik, B. and Jornvall, H. (1985b) Structural evidence for three different types of glutathione transferase in human tissues. FEBS Lett., 182: 319-322.

Allen, G. (1981) Sequencing of proteins and peptides. In: Work, T.S. and Work, E. (ed.) Laboratory Techniques in Biochemistry and Molecular Biology. Elsevier Biomedical Press, Oxford, pp. 109-121.

Ambler, R.P. (1963) Pseudomonas cytochrome C-551. Biochem. J., 89: 349-378.

Ambler, R.P. and Brown, L.H. (1967) The amino acid sequence of Pseudomonas fluorescens azurin. Biochem. J., 104: 784-825.

Arias, I.M. (1979) Ligandin, review and update of a multifunctional protein. Med. Biology, 57: 328-334.

Asaoka, K. (1984) Affinity purification and characterization of glutathione S-transferases from bovine liver. J. Biochem., 95: 685-696.

Askelof, P., Guthenberg, C., Jakobson, I. and Mannervik, B. (1975) Purification and characterization of two glutathione S-aryltransferase activities from rat liver. Biochem. J., 147: 513-522.

Awasthi, Y.C. and Singh, S.V. (1985) Subunit structure of human and rat glutathione S-transferases. Comp. Biochem. Physiol., 82B: 17-23.

Awasthi, Y.C., Dao, D.D. and Saneto, R.P. (1980) Interrelationship between anionic and cationic forms of glutathione S-transferases of human liver. Biochem. J., 191: 1-10.

Bach, M.K., Brashler, J.R. and Morton, Jr., D.R. (1984) Solubilization and characterization of the leukotriene C_4 synthetase of rat basophil leukemia cells: A novel particulate glutathione S-transferase. Arch. Biochem. Biophys., 230: 455-465.

Barnes, M.M., James, S.P. and Wood, P.B. (1959) The formation of mercapturic acids. Formation of mercapturic acid and the levels of glutathione in tissues. Biochem. J., 71: 680-690.

Bass, N.M., Kirsch, R.E., Tuff, S.A., Marks, I. and Saunders, S.J. (1977) Ligandin heterogeneity: evidence that the two non-identical subunits are the monomers of two distinct proteins. Biochim. Biophys. Acta, 492: 163-175.

Batist, G., Tulpule, A., Sinha, B.K., Katki, A.G., Myers, C.E. and Cowan, K.H. (1986) Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. J. Biol. Chem., 261: 15544-15549.

Baumann, E. and Preusse, C. (1879) Ueber bromophenylmercaptursaure. Ber. Dtsch. Chem. Ges., 12: 806-810.

Beale, D., Ketterer, B., Carne, T., Meyer, D., Taylor, J.B. (1982) Evidence that the Ya and Yc subunits of glutathione transferase B (ligandin) are the products of separate genes. Eur. J. Biochem., 126: 459-463.

Beale, D., Meyer, D.J., Taylor, J.B. and Ketterer, B. (1983) Evidence that the Yb subunits of hepatic glutathione transferases represent two different but related families of polypeptides. Eur. J. Biochem., 137: 125-129.

Beckett, G.J. and Hayes, J.D. (1984) Development of specific radioimmunoassays for the measurement of human hepatic basic and N/A2b glutathione S-transferases. Clin. Chim. Acta, 141: 267-273.

Beckett, G.J., Dyson, E.H., Chapman, B.J., Templeton, A.J. and Hayes, J.D. (1985) Plasma glutathione S-transferase measurements by radioimmunoassay: a sensitive index of hepatocellular damage in man. Clin. Chim. Acta, 146: 11-19.

Benson, J.V. and Patterson, J.A. (1965) Accelerated automatic chromatographic analysis of amino acids on a spherical resin. Anal. Chem., 37: 1108-1110.

Benson, A.M. and Talalay, P. (1976) Role of reduced glutathione in the Δ^5-3-ketosteroid isomerase reaction of liver. Biochem. Biophys. Res. Commun., 69: 1073-1079.

Benson, A.M., Talalay, P., Keen, J.H. and Jakoby, W.B. (1977) Relationship between the soluble glutathione-dependent Δ^5-3-ketosteroid isomerase and the glutathione S-transferases of the liver. Proc. Natl. Acad. Sci., USA, 74: 158-162.

Bhargava, M.M., Listowsky, I. and Arias, I.M. (1978) Studies on subunit structure and evidence that ligandin is a heterodimer. J. Biol. Chem., 253: 4116-4119.

Board, P.G. (1981a) Biochemical genetics of glutathione S-transferase in man. Am. J. Hum. Genet., 33: 36-43.

Board, P.G. (1981b). Gene deletion and partial deficiency of the glutathione S-transferase (Ligandin) system in man. FEBS Lett., 135: 12-14.

Booth, J., Boyland, E., Sims, P. (1961) An enzyme from rat liver catalysing conjugations with glutathione. Biochem. J., 79: 516-524.

Boyer, T.D., Kenney, W.C. and Zakim, D. (1983) Structural, functional and hybridization studies of glutathione S-transferases of rat liver. Biochem. Pharmacol., 32: 1843-1850.

Boyer, T.D., Vessey, D.A., Holcomb, C. and Saley N. (1984) Studies of the relationship between catalytic activity and binding of non-substrate ligands by the glutathione S-transferase. Biochem. J., 217: 179-185.

Boyland, E. and Chasseaud, L.F. (1969) The role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis. Adv. Enzymol., 32: 172-219.

Bradford, M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Bray, H.G., Franklyn, T.J. and James, S.P. (1959) The formation of mercapturic acids. The possible role of glutathione. Biochem. J., 77: 690-696.

Burk, R.F., Nishiki, K., Lawrence, R.A. and Chance, B. (1978) Peroxide removed by selenium-dependent and selenium-independent glutathione peroxidases in haemoglobin-free perfused rat liver. J. Biol. Chem., 253: 43-46.

Cagan, L.M., Pisano, J.J., Ketley, J.N., Habig, W.H. and Jakoby, W.B. (1975) The conjugation of prostaglandin A_1 and glutathione catalysed by homogenous glutathione S-transferases from human and rat liver. Biochim. Biophys. Acta, 398: 205-208.

Carnegie, P.R. (1963) Isolation of a homologue of glutathione and other acidic peptides from seedlings of Phaseolus aureus. Biochem. J., 89: 459-470.

Chang, C.K., Clark, A.G., Fieldes, A. and Pound, S. (1981) Some properties of a glutathione S-transferase from the larvae of Galleria mellonella. Insect. Biochem., 11: 179-186.

Chasseaud, L.F. (1976) Conjugation with glutathione and mercapturic acid excretion. In: Arias, I.M. and Jakoby, W.D. (ed.) Glutathione: Metabolism and Function. Raven Press, New York, pp. 77-114.

Chasseaud, L.F. (1979) The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents. Adv. Cancer Res., 29: 175-274.

Chaudhari, A., Anderson, M.W. and Eling, T.E. (1978) Conjugation of 15-keto-prostaglandins by glutathione S-transferases. Biochim. Biophys. Acta, 531: 56-64.

Clark, A.G., Dick, D.L. and Smith J.N. (1984) Kinetic studies of glutathione S-transferase from the larvae of Costelytra zealandica. Biochem. J., 217: 51-58.

Cleland, W.W. (1963) The kinetics of enzyme-catalyzed reactions with two or more substrates or products. Biochim. Biophys. Acta, 67: 104-137.

Coles, B., Meyer, D.J., Ketterer, B., Stanton, C.A. and Garner, R.C. (1985) Studies on the detoxication of microsomally-activated aflatoxin B_1 by glutathione and glutathione transferase in vitro. Carcinogenesis, 6: 693-697.

Combes, B. and Stakelum, G.S. (1961) A liver enzyme that conjugates sulphobromophthalein sodium with glutathione. J. Clin. Invest., 40: 981-988.

Daniel, V., Sarid, S., Bar-Nun, S. and Litwack, G. (1983) Rat ligandin mRNA molecular cloning and sequencing. Arch. Biochem. Biophys., 227: 266-271.

Dao, D.D., Partridge, C.A. and Awasthi, Y.C. (1982) Subunit structure of glutathione S-transferases of human liver and placenta. I.R.C.S. Medical Science: Biochemistry, 10: 175.

Dao, D.D., Partridge, C.A., Kurosky, A. and Awasthi, Y.C. (1984) Human glutathione S-transferases. Characterisation of the anionic forms from lung and placenta. Biochem. J., 221: 33-41.

Ding, V.D.-H. and Pickett, C.D. (1985). Translational regulation of rat liver glutathione S-transferase genes by phenobarbital and 3-methylcholanthrene. Arch. Biochem. Biophys., 240: 553-559.

Ding, G.J.-F., Lu, A.Y.H. and Pickett, C.B. (1985) Rat liver glutathione S-transferases. Nucleotide sequence analysis of a Yb_1 cDNA clone and prediction of the complete amino acid sequence of the Yb_1 subunit. J. Biol. Chem., 260: 13268-13271.

Ding, G.J.-F., Ding, V.D.-H., Rodkey, J.A., Bennett, C.D., Lu, A.Y.H. and Pickett, C.B. (1986) Rat liver glutathione S-transferases. DNA sequence analysis of a Yb_2 cDNA clone and regulation of the Yb_1 and Yb_2 mRNAs by phenobarbital. J. Biol. Chem., 261: 7952-7957.

Dixon, M. and Webb, E.C. (1979) Fractional precipitation by salts. Enzymes, 3rd ed., Longman Group Ltd., London, pp. 31-33.

Douglas, K.T. (1987) Mechanism of action of glutathione-dependent enzymes. Adv. Enzymol., 59: 103-167.

Elce, J.S. (1972) Metabolism of a glutathione conjugate of 2-hydroxyoestradiol -17β in the adult male rat. Biochem. J., 126: 1067-1071.

Eriksson, S., Askelof, P., Axelsson, K., Carlberg, I., Guthenberg, C. and Mannervik, B. (1974) Resolution of glutathione-linked enzymes in rat liver and evaluation of their contribution to disulphide reduction via thiol-disulphide interchange. Acta Chem. Scand., B28: 922-930.

Eriksson, L.C., Shorma, R.N., Roomi, R.K., Farber, E. and Murray, R.K. (1983) A characteristic electrophoretic pattern of cytosolic polypeptides from hepatocyte nodules generated during liver carcinogenesis in several models. Biochem. Biophys. Res. Commun., 117: 740-745.

Esterbauer, H., Cheesema, K.H., Dianzani, M.U., Poi, I.G. and Slater, T.F. (1982) Separation and characterization of the aldehydic products of lipid peroxidation stimulated by ADP-Fe^{2+} in rat liver microsomes. Biochem. J., 208: 129-140.

Farber, E. (1984) Pre-cancerous steps in carcinogenesis. Their physiological adaptive nature. Biochim. Biophys. Acta, 738: 171-180.

Fjellstedt, T.A., Allen, R.H., Duncan, B.K. and Jakoby, W.B. (1973) Enzymatic conjugation of epoxides with glutathione. J. Biol. Chem., 248: 3702-3707.

Frey, A.B., Friedberg, T., Oesch, F. and Kreibich, G. (1983) Studies on the subunit composition of rat liver glutathione S-transferases. J. Biol. Chem., 258: 11321-11325.

Friedberg, T., Milbert, U., Bentley, P., Guenther, T.M. and Oesch, F. (1983) Purification and characterization of a new cytosolic glutathione S-transferase (glutathione S-transferase X) from rat liver. Biochem. J., 215: 617-625.

Gilbert, H.F. (1982) Biological disulphides: The third messenger. J. Biol. Chem., 257: 12086-12091.

Gillham, B. (1971) The reaction of aralkyl sulphate esters with glutathione catalysed by rat liver preparations. Biochem. J., 121: 667-672.

Gillham, B. (1973) The mechanism of the reaction between glutathione and 1-menaphthyl sulphate catalysed by a glutathione S-transferase from rat liver. Biochem. J., 135: 797-804.

Glatt, H., Friedberg, T., Grover, P.L., Sims, P. and Oesch, F. (1983) Inactivation of a diol-epoxide and a K-region epoxide with high efficiency by glutathione transferase X. Cancer Res., 43: 5713-5717.

Glazer, A.N., Delange, R.J. and Sigman, D.S. (1975) Chemical modification of proteins. In: Work, T.S. and Work, E. (ed.) Laboratory Techniques in Biochemistry and Molecular Biology. Elsevier Biomedical Press, Oxford, pp. 13-64.

Grover, P.L. (1982) Glutathione S-transferases in detoxification. Biochem. Soc. Trans., 10: 80-82.

Grover, P.L. and Sims, P. (1964) Conjugation with glutathione. Distribution of glutathione S-transferase in vertebrate species. Biochem. J., 90: 603-606.

Guthenberg, C. and Mannervik, B. (1981) Glutathione S-transferase (transferase π) from human placenta is identical or closely related to glutathione S-transferase (transferase ρ) from erythrocytes. Biochim. Biophys. Acta, 661: 255-260.

Guthenberg, C., Akerfeldt, K. and Mannervik, B. (1979) Purification of glutathione S-transferase from human placenta. Acta Chem. Scand., B33: 595-596.

Habig, W.H. (1983) Glutathione S-transferases: Versatile enzymes of detoxication. In: Nygaard, O.F. and Simic, M.G. (ed.) Radioprotectors and Anticarcinogens. Academic Press, New York, pp. 169-190.

Habig, W.H. and Jakoby, W.B. (1981) Assays for differentation of glutathione S-tranferases. Methods Enzymol., 77: 398-405.

Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M. and Jakoby, W.B. (1974a) The identity of glutathione S-tranferase B with ligandin, a major binding protein of liver. Proc. Natl. Acad. Sci. USA, 71: 3879-3882.

Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974b) Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. J. Biol. Chem., 249: 7130-7139.

Habig, W.H., Keen, J.H. and Jakoby, W.B. (1975) Glutathione S-transferase in the formation of cyanide from organic thiocyanates and as an organic nitrate reductase. Biochem. Biophys. Res. Commun., 64: 501-506.

Habig, W.H., Kamisaka, K., Ketley, J.N., Pabst, M.J., Arias, I.M. and Jakoby, W.B. (1976a) The human hepatic glutathione S-transferases. In: Arias, I.M. and Jakoby, W.B. (ed.) Glutathione : Metabolism and Function. Raven Press, New York, pp. 225-232.

Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1976b) Glutathione S-transferase AA from rat liver. Arch. Biochem. Biophys., 175: 710-716.

Hales, B.F., Jaeger, V. and Neims, A.H. (1978) Isoelectric focusing of glutathione S-transferases from rat liver and kidney. Biochem. J., 175: 937-943.

Halliwell, B. and Gutteridge, J.M.C. (1985a) Free radicals in biology and medicine. Clarendon Press, Oxford, pp. 246-247.

Halliwell, B. and Gutteridge, J.M.C. (1985b) Free radicals in biology and medicine. Clarendon Press, Oxford, p.182.

Hammerstrom, S. (1983) Leukotrienes. Ann. Rev. Biochem., 52: 355-377.

Harris, J.I. and Hindley, J. (1965) The protein subunit of turnip yellow mosaic virus. J. Mol. Biol., 13: 894-913.

Hayakawa, I., Myokel, Y., Yagi, H. and Jerina, D.M. (1977) Purification and some properties of glutathione S-epoxide transferase from guinea pig liver. J. Biochem., 82: 407-415.

Hayes, J.D. (1983) Rat liver glutathione S-transferases. A study of the structure of the basic YbYb-containing enzymes. Biochem. J., 213: 625-633.

Hayes, J.D. (1984) Purification and characterization of glutathione S-transferases P, S and N. Biochem. J., 224: 839-852.

Hayes, J.D. (1986) Purification and physical characterization of glutathione S-transferase K. Biochem. J., 233: 789-798.

Hayes, J.D. and Chalmers, J. (1983) Bile acid inhibition of basic and neutral glutathione S-transferases in rat liver. Biochem. J., 215: 581-588.

Hayes, J.D. and Clarkson, G.H.D. (1982) Purification and characterization of three forms of glutathione S-transferase A. Biochem. J., 207: 459-470.

Hayes, J.D. and Mantle, T.J. (1986a) Inhibition of hepatic and extrahepatic glutathione S-transferases by primary and secondary bile acids. Biochem. J., 233: 407-415.

Hayes, J.D. and Mantle, T.J. (1986b) Use of immuno-blot techniques to discriminate between the glutathione S-transferase Yf, Yk, Ya, Yn/Yb and Yc subunits and to study their distribution in extrahepatic tissues. Biochem. J., 233: 779-788.

Hayes, J.D. and Mantle, T.J. (1986c) Anomalous electrophoretic behaviour of the glutathione S-transferase Ya and Yk subunits isolated from man and rodents. Biochem. J., 237: 731-740.

Hayes, J.D., Strange, R.C. and Percy-Robb, I.W. (1979) Identification of two lithocholic acid-binding proteins. Separation of ligandin from glutathione S-transferase B. Biochem. J., 181: 699-708.

Hayes, J.D., Strange, R.C. and Percy-Robb, I.W. (1981) A study of the structures of the YaYa and YaYc glutathione S-transferases from rat liver cytosol. Biochem. J., 197: 491-502.

Hayes, J.D., Gilligan, D., Chapman, B.J. and Beckett, G.J. (1983) Purification of human hepatic glutathione S-transferases and the development of a radioimmunoassay for their measurement in plasma. Clin. Chim. Acta, 134: 107-121.

Hirom, P.C., Milburn, P., Smith, R.L. and Williams, R.T. (1972) Species variations in the threshold molecular weight factor for the biliary excretion of organic ions. Biochem. J., 129: 1071-1077.

Hirs, C.H.W. (1967) Performic acid oxidation. Methods Enzymol., 11: 197-199.

Homma, H. and Listowsky, I. (1985) Identification of Yb-glutathione S-transferase as a major rat liver protein labelled with dexamethasone 21-methanesulfonate. Proc. Natl. Acad. Sci., USA, 82: 7165-7169.

Jaffe, M. (1879) Ueber die nach einfuhrung von bromobenzol und chlorobenzol in organismus entsthenden schwefelthaltigen sauren. Ber. Dtsch. Chem. Ges., 12: 1092-1098.

Jakobson, I., Askelof, P., Warholm, M. and Mannervik, B. (1977) A steady-state-kinetic random mechanism for glutathione S-transferase A from rat liver. Eur. J. Biochem., 77: 253-262.

Jakobson, I., Warholm, M. and Mannervik, B. (1979a) Multiple inhibition of glutathione S-transferase A from rat liver by glutathione derivatives: Kinetic analysis supporting a steady-state random sequential mechanism. Biochem. J., 177: 861-868.

Jakobson, I., Warholm, M. and Mannervik, B. (1979b) The binding of substrates and a product of the enzymatic reaction to glutathione S-transferase A. J. Biol. Chem., 254: 7085-7089.

Jakoby, W.B. (1978a) The glutathione S-transferases, a group of multifunctional detoxification proteins. Adv. Enzymol., 46: 383-414.

Jakoby, W.B. (1978b) The glutathione transferases in detoxification. Proceedings in Life Sci., 159-163.

Jakoby, W.B. and Habig, W.H. (1980) Glutathione transferases. In: Jakoby, W.B. (ed.). Enzymatic Basis of Detoxication, Vol. 2. Academic Press, New York, pp. 63-95.

Jakoby, W.B. and Keen, J.H. (1977) Triple-threat in detoxification. Glutathione S-transferases. Trends Biochem. Sci., 2: 229-231.

Jakoby, W.B., Habig, W.H., Keen, J.H., Ketley, J.N. and Pabst, M.J. (1976a) Glutathione S-transferases: Catalytic aspects. In: Arias, I.M. and Jakoby, W.B. (ed.). Glutathione: Metabolism and Function. Raven Press, New York, pp. 189-202.

Jakoby, W.B., Ketley, J.N. and Habig, W.H. (1976b) Rat glutathione S-transferases: Binding and physical properties. In: Arias, I.M. and Jakoby, W.B. (ed.) Glutathione: Metabolism and Function. Raven Press, New York, pp. 213-220.

Jakoby, W.B., Ketterer, B. and Mannervik, B. (1984) Glutathione transferases: Nomenclature. Biochem. Pharmacol., 33: 2539-3540.

Jellinck, P.H., Lewis, J. and Boston, F. (1967) Further evidence for the formation of an estrogen-peptide conjugate by rat liver in vitro. Steroids, 10: 329-346.

Jensson, H., Eriksson, L.C. and Mannervik, B. (1985) Selective expression of glutathione transferase isoenzymes in chemically-induced pre-neoplastic rat hepatocyte nodules. FEBS Lett., 187: 115-120.

Jensson, H., Guthenberg, C., Alin, P. and Mannervik, B. (1986) Rat glutathione transferase 8-8, an enzyme efficiently detoxifying 4-hydroxyalk-2-enals. FEBS Lett., 203: 207-209.

Kalinyak, J.E. and Taylor, J.M. (1982) Rat glutathione S-transferase. Cloning of double-stranded cDNA and induction of its mRNA. J. Biol. Chem., 257: 523-530.

Kamisaka, K., Habig, W.H., Ketley, J.N., Arias, I.M. and Jakoby, W.B. (1975) Multiple forms of human glutathione S-transferase and their affinity for bilirubin. Eur. J. Biochem., 60: 153-161.

Kaplowitz, N. (1980) Physiological significance of glutathione S-transferases. Am. J. Physiol., 239: G439-G444.

Kaplowitz, N., Percy-Robb, I.W. and Javitt, N.B. (1973) Role of hepatic anion-binding protein in bromsulphthalein conjugation. J. Exp. Med., 138: 483-487.

Kaplowitz, N., Clifton, G., Kuhlenkamp, J. and Wallin, J.D. (1976) Comparison of renal and hepatic glutathione S-transferases of the rat. Biochem. J., 158: 243-248.

Keen, J.H. and Jakoby, W.B. (1978) Glutathione transferases: catalysis of nucleophilic reactions of glutathione. J. Biol. Chem., 253: 5654-5657.

Keen, J.H., Habig, W.H. and Jakoby, W.B. (1976) Mechanism of the several activities of the glutathione S-transferases. J. Biol. Chem., 251: 6183-6188.

Ketley, J.N., Habig, W.H. and Jakoby, W.B. (1975) Binding of non-substrate ligands to the glutathione S-transferases. J. Biol. Chem., 250: 8670-8673.

Ketterer, B., Ross-Mansell, P. and Whitehead, J.K. (1967) The isolation of carcinogen-binding protein from livers of rats given 4-dimethylamino-azobenzene. Biochem. J., 103: 316-324.

Ketterer, B., Tipping, E., Meuwissen, J. and Beale, D. (1975) Ligandin. Biochem. Soc. Trans., 3: 626-630.

Ketterer, B., Coles, B. and Meyer, D.J. (1983) The role of glutathione in detoxication. Environ. Health Perspect., 49: 59-69.

Kitahara, A. and Sato, K. (1981) Immunological relationships among subunits of glutathione S-transferases A, AA, B, ligandin and hybrid formation between AA and ligandin by guanidine hydrochloride. Biochem. Biophys. Res. Commun., 103: 943-950.

Kitahara, A., Satoh, K. and Sato, K. (1983) Properties of the increased glutathione S-transferase A form in rat preneoplastic hepatic lesions induced by chemical carcinogens. Biochem. Biophys. Res. Commun., 112: 20-28.

Kitahara, A., Satoh, K., Nishimura, K., Ishikawa, T., Ruike, K., Sato, K., Tsuda, H. and Ito, N. (1984) Changes in molecular forms of rat hepatic glutathione S-transferase during chemical hepatocarcinogenesis. Cancer Res., 44: 2698-2703.

Kodate, C., Fukushi, A., Narita, T., Kudo, H., Soma, Y. and Sato, K. (1986) Human placental form of glutathione S-transferase (GST π) as a new immunohistochemical marker for human colonic carcinoma. Jpn. J. Cancer Res. (Gann), 77: 226-299.

Koskelo, K. (1983) Isoelectric focusing of glutathione S-transferases: Comparison of the acidic transferases from human liver, kidney, lung, spleen and placenta. Scand. J. clin. Lab. Invest., 43: 133-139.

Koskelo, K, and Icen, A. (1984) Chromatofocusing of glutathione S-transferases from human kidney. Scand. J. clin. Lab. Invest., 44: 159-162.

Koskelo, K. and Valmet, E. (1980) Acid glutathione S-transferase from human liver : preliminary report. Scand. J. clin. Lab. Invest., 40: 179-184.

Koskelo, K., Valmet, E. and Tenhunen, R. (1981) Purification and characetization of an acid glutathione S-transferase from human lung. Scand. J. clin. Lab. Invest., 41: 683-689.

Kosower, N.S. and Kosower, E.M. (1976) Functional aspects of glutathione disulphide and hidden forms of glutathione. In: Arias, I.M. and Jakoby, W.B. (ed.) Glutathione: Metabolism and Function. Raven Press, New York, pp. 159-174.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 227: 680-685.

Lai, H.-C.J., Li, N., Weiss, M.J., Reddy, C.C. and Tu, C.-P.D. (1984) The nucleotide sequence of a rat liver glutathione S-transferase subunit cDNA clone. J. Biol. Chem., 259: 5536-5542.

Lawrence, R.A. and Burk, R.F. (1976) Glutathione peroxidase activity in selenium deficient rat liver. Biochem. Biophys. Res. Commun., 71: 952-958.

Levi, A.J., Gatmaitan, Z. and Arias, I.M. (1969) The role of two hepatic proteins (Y and Z) in the transfer of sulfobromophthalein (BSP) and bilirubin from plasma into the liver. J. Clin. Invest., 48: 2156-2167.

Levi, A.J., Gatmaitan, Z. and Arias, I.M. (1970) Deficiency of hepatic organic anion binding protein, impaired organic anion uptake by liver and "physiologic" jaundice in newborn monkeys. N. Engl. J. Med., 28: 1136-1139.

Levine, R.I., Reyes, H., Levi, A.J., Gatmaitan, Z. and Arias, I.M. (1971) Phylogenetic study of organic anion transfer from plasma into liver. Nature (New Biology), London, 231: 277-279.

Li, N.-Q., Reddanna, P., Thyagaraju, K., Reddy, C.C. and Tu, C.-P.D. (1986) Expression of glutathione S-transferases in rat brains. J. Biol. Chem., 261: 7596-7599.

Listowsky, I., Kamisaka, K., Ishitami, K. and Arias, I.M. (1976) Structure and properties of ligandin. In: Arias, I.M. and Jakoby, W.B. (ed.) Glutathione: Metabolism and Function. Raven Press, New York, pp. 233-239.

Litwack, G., Ketterer, B. and Arias, I.M. (1971) Ligandin : a hepatic protein which binds steroids, bilirubin, carcinogens and a number of exogenous anions. Nature, (London), 234: 466-467.

Mangold, J.B. and Abdel-Monem, M.M. (1980) Stereoselectivity of the glutathione S-transferase conjugation of aralkyl halides. Biochem. Biophys. Res. Commun., 96: 333-340.

Mangold, J.B. and Abdel-Monem, M.M. (1983) Stereochemical aspects of conjugation reactions catalysed by rat liver glutathione S-transferase isoenzymes. J. Med. Chem., 26: 66-71.

Mannervik, B. (1985) The isoenzymes of glutathione transferase. Adv. Enzymol., 57: 357-417.

Mannervik, B. and Guthenberg, C. (1981) Glutathione transferase (human placenta). Methods Enzymol., 77: 231-235.

Mannervik, B. and Jensson, H. (1982) Binary combinations of four protein subunits with different catalytic specificities explain the relationship between six basic glutathione S-transferases in rat liver cytosol. J. Biol. Chem., 257: 9909-9912.

Mannervik, B., Jensson, H., Alin, P., Orning, L. and Hammarstrom, S. (1984) Transformation of leukotriene A_4 methyl ester to leukotriene C_4 monomethyl ester by cytosolic rat glutathione transferases. FEBS Lett., 174: 289-293.

Mannervik, B., Alin. P. Guthenberg, C., Jensson, H., Tahir, M.K., Warholm, M. and Jornvall, H. (1985) Identification of three classes of cytosolic glutathione transferase common to several mammalian species: Correlation between structural data and enzymatic properties. Proc. Natl. Acad. Sci. USA., 82: 7202-7206.

Marcus, C.J., Habig, W.H. and Jakoby, W.B. (1978) Glutathione transferase from human erythrocytes. Non-identity with the enzymes from liver. Arch. Biochem. Biophys., 188: 287-293.

Maruyama, H., Arias, I.M. and Listowsky, I. (1984) Distinctions between the multiple cationic forms of rat liver glutathione S-transferase. J. Biol. Chem., 259: 12444-12448.

Meister, A. (1983) Selective modification of glutathione metabolism. Science, 220: 472-477.

Meister, A. and Anderson, M.E. (1983) Glutathione. Ann. Rev. Biochem., 52: 711-760.

Meister, A. and Tate, S.S. (1976) Glutathione and related γ-glutamyl compounds: Biosynthesis and utilization. Ann. Rev. Biochem., 45: 559-604.

Meyer, D.J. and Ketterer, B. (1982) 5,6-epoxy-cholestan-3β-ol (cholesterol α-oxide): A specific substrate for rat liver glutathione transferase B. FEBS Lett., 150: 499-501.

Meyer, D.J., Christodoulides, L.G., Tan, K.H. and Ketterer, B. (1984) Isolation, properties and tissue distribution of rat glutathione transferase E. FEBS Lett., 173: 327-330.

Mills, G.C. (1957) Glutathione peroxidase, an erthyrocyte enzyme which protects haemoglobin from oxidative breakdown. J. Biol. Chem., 229: 189-197.

Milstein, C.P. and Milstein, C. (1968) A tryptic peptide containing a unique serine phosphate residue in rabbit phosphoglucomutase. Biochem. J., 109: 93-99.

Moore, M.A., Nakagawa, K., Satoh, K., Ishikawa, T. and Sato, K. (1987) Single GST-P positive liver cells: putative initiated hepatocytes. Carcinogenesis, 8: 483-486.

Morey, K.S. and Litwack, G. (1969) Isolation and properties of cortisol metabolite binding proteins of rat liver cytosol. Biochemistry, 8: 4812-4821.

Morgenstern, R. and De Pierre, J.W. (1983) Microsomal glutathione transferase. Eur. J. Biochem., 134: 591-597.

Motoyama, N., Hayaoka, T., Nomura, K. and Dauterman, W.C. (1980) Multiple factors for organophosphorous resistance in the housefly, Musca domestica L. J. Pesticide Sci., 5: 393-402.

Nemoto, N., Gelboin, H.W., Habig, W.H., Ketley, J.N. and Jakoby, W.B. (1975) K-region benzo[a]pyrene-4,5-oxide is conjugated by homogeneous glutathione S-transferases. Nature, 255: 512.

Ostlund Farrants, A.-K., Meyer, D.J., Coles, B., Southam, C., Aitken, A., Johnson, P.J. and Ketterer, B. (1987) The separation of glutathione transferase subunits by using reverse-phase high-pressure liquid chromatography. Biochem. J., 245: 423-428.

Pabst, M.J., Habig, W.H. and Jakoby, W.B. (1973) Mercapturic acid formation, the several glutathione transferases of rat liver. Biochem. Biophys. Res. Commun., 52: 1123-1128.

Pabst, M.J., Habig, W.H. and Jakoby, W.B. (1974) Glutathione S-transferase A: A novel kinetic mechanism in which the major reaction pathway depends on substrate concentration. J. Biol. Chem., 249: 7140-7148.

Partridge, C.A., Dao, D.D. and Awasthi, Y.C. (1984) Glutathione S-transferases of lung : purification and characterization of human lung glutathione S-transferases. Lung, 162: 27-36.

Pattinson, N.R. (1981) Cholic acid binding by anionic glutathione S-transferase from human liver cytosol. Biochem. Biophys. Res. Commun., 102: 403-410.

Pearson, W.R., Windle, J.J., Morrow, J.F., Benson, A.M. and Talalay, P. (1983) Increased synthesis of glutathione S-transferase in response to anticarcinogenic anti-oxidants. J. Biol. Chem., 258: 2052-2062.

Pemble, S.E., Taylor, J.B. and Ketterer, B. (1986) Tissue distribution of rat glutathione transferase subunit 7, a hepatoma marker. Biochem. J., 240: 885-889.

Pickett, C.B., Telakowski-Hopkins, C.A., Ding, G.J.-F., Argenbright, L. and Lu, A.Y.H. (1984) Rat liver glutathione S-transferases. Complete nucleotide sequence of a glutathione S-transferase mRNA and the regulation of the Ya, Yb and Yc mRNAs by phenobarbital. J. Biol. Chem., 259: 5182-5188.

Prohaska, J.R. and Ganther, H.E. (1977) Glutathione peroxidase activity of glutathione S-transferases purified from rat liver. Biochem. Biophys. Res. Commun., 76: 437-445.

Ramage, P.I. and Nimmo, I.A. (1983) The purification of the hepatic glutathione S-transferases of rainbow trout by glutathione affinity chromatography alters their isoelectric behaviour. Biochem. J., 211: 523-526.

Reddy, C.C., Tu, C.-P., Burgess, J.R., Ho, C.-Y., Scholz, R.W. and Massaro, E.J. (1981) Evidence for the occurrence of selenium-independent glutathione peroxidase activity in rat liver microsomes. Biochem. Biophys. Res. Commun., 101: 970-978.

Reddy, C.C., Burgess, J.R. and Tu, C.-P.D. (1983) Isolation and characterization of an anionic glutathione S-transferase from rat liver cytosol. Biochem. Biophys. Res. Commun., 3: 840-846.

Reddy, C.C., Li, N. and Tu, C.-P.D. (1984) Identification of a new glutathione S-transferase from rat liver cytosol. Biochem. Biophys. Res. Commun., 121: 1014-1020.

Reyes, H., Levi, A.J., Gatmaitan, Z. and Arias, I.M. (1971) Studies of Y and Z, two hepatic cytoplasmic organic anion-binding proteins: Effect of drugs, chemicals, hormones and cholestasis. J. Clin. Invest., 50: 2242-2252.

Rhoads, D.M., Zarlengo, R.P. and Tu, C.-P.D. (1987) The basic glutathione S-transferases from human livers are products of separate genes. Biochem. Biophys. Res. Commun., 145: 474-481.

Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. and Sato, K. (1985) Purification, induction and distribution of placental glutathione transferase: A new marker enzyme for pre-neoplastic cells in the rat chemical carcinogenesis. Proc. Natl. Acad. Sci., USA, 82: 3964-3968.

Scully, N.C. and Mantle, T.J. (1981) Tissue distribution and subunit structures of the multiple forms of glutathione S-transferase in the rat. Biochem. J., 193: 367-370.

Sheehan, D. and Mantle, T.J. (1984) Evidence for two forms of ligandin (YaYa dimers of glutathione S-transferase) in rat liver and kidney. Biochem. J., 218: 893-897.

Simons, P.C. and Vander Jagt, D.L. (1977) Purification of glutathione S-transferases from human liver by glutathione-affinity chromatography. Anal. Biochem., 82: 334-341.

Simons, P.C. and Vander Jagt, D.L. (1980) Bilirubin binding to human liver ligandin (Glutathione S-transferase). J. Biol. Chem., 255: 4740-4744.

Singh, S.V., Theodore, C., Hong, T.D., Awasthi, Y.C. (1984) Glutathione S-transferases of human liver. Amer. J. Hum. Genet., 36: suppl. 054.

Singh, S.V., Dao, D.D., Partridge, C.A., Theodore, C., Srivastava, S.K. and Awasthi, Y.C. (1985) Different forms of human liver glutathione S-transferases arise from dimeric combinations of at least four immunologically and functionally distinct subunits. Biochem. J., 232: 781-790.

Sluyterman, L.A.AE. (1982) Chromatofocusing: A preparative protein separation method. Trends Biochem. Sci., 7: 168-170.

Smith, G.J., Ohl, V.S. and Litwack, G. (1977) Ligandin, the glutathione S-transferases and chemically-induced hepatocarcinogenesis: A review. Cancer Res., 37: 8-14.

Soma, Y., Satoh, K. and Sato, K. (1986) Purification and subunit-structural and immunological characterization of five glutathione S-transferases in human liver and the acidic form as a hepatic tumor marker. Biochim. Biophys. Acta, 869: 247-258.

Stekol, J.A. (1938) Studies on the mercapturic acid synthesis in animals. The conversion of benzyl chloride and S-benzylcysteine into benzylmercapturic acid in the organs of the dog, rabbit and rat. J. Biol. Chem., 124: 129-132.

Strange, R.C., Cramb, R., Hayes, J.D. and Percy-Robb, I.W. (1977) Partial purification of two lithocholic acid-binding proteins from rat liver 100 000 g supernatants. Biochem. J. , 115: 425-429.

Strange, R.C., Faulder, C.G., Davis, B.A., Hume, R., Brown, J.A.H., Cotton, W. and Hopkinson, D.A. (1984) The human glutathione S-transferases: studies on the tissue distribution and genetic variation of the GST1, GST2 and GST3 isozymes. Ann. Hum. Genet., 48: 11-20.

Sugioka, Y., Kano, T., Okuda, A., Sakai, M., Kitagawa, T. and Marumatsu, M. (1985) Cloning and nucleotide sequence of rat glutathione S-transferase P cDNA. Nuc. Acid. Res., 13: 6049-6057.

Sugiyama, Y., Stolz, A., Sugimoto, M. and Kaplowitz, N. (1984) Evidence for a common high affinity binding site on glutathione S-transferase B for lithocholic acid and bilirubin. J. Lipid Res., 25: 1177-1183.

Tahir, M.K., Guthenberg, C. and Mannervik, B. (1985) Inhibitors for distinction of three types of human glutathione transferase. FEBS lett, 181: 249-252.

Tan, H.K., Meyer, D.J. and Ketterer, B. (1983) Inhibition of lipid peroxidation in rat liver microsomes by glutathione S-transferase B. Biochem. Soc. Trans., 11: 308-309.

Tateoka, N., Tsuchida, S., Soma, Y. and Sato, K. (1987) Purification and characterization of glutathione S-transferases in human kidney. Clin. Chim. Acta, 166: 207-218.

Taylor, J.B., Craig, R.K., Beale, D. and Ketterer, B. (1984) Construction and characterization of a plasmid containing complementary DNA to mRNA encoding the N-terminal amino acid sequence of the rat glutathione transferase Ya subunit. Biochem. J., 219: 223-231.

Telakowski-Hopkins, C.A., Rodkey, J.A., Bennett, C.D., Lu, A.Y.H. and Pickett, C.B. (1985) Rat liver glutathione S-transferases: Construction of a cDNA clone complementary to a Yc mRNA and prediction of the complete amino acid sequence of a Yc subunit. J. Biol., Chem., 260: 5820-5825.

Tipping, E., Ketterer, B., Christodoulides, L. and Enderby, G. (1976) Spectroscopic studies of the binding of bilirubin by ligandin and amino-dye binding protein A. Biochem. J., 157: 211-216.

Towbin, H., Staehlin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide-gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci., USA, 76:

Tu, C.-P.D. and Qian, B. (1986) Human liver glutathione S-transferases : complete primary sequence of an Ha subunit cDNA. Biochem. Biophys. Res. Commun., 141: 229-237.

Tu, C.-P.D., Weiss, M.J., Karakawa, W.W. and Reddy, C.C. (1982a) Cloning and sequence analysis of a cDNA plasmid for one of the rat liver glutathione S-transferase subunits. Nuc. Acids Res., 10: 5407-5419.

Tu, C.-P.D., Weiss, M.J. and Reddy, C.C. (1982b) Subunit composition of rat liver glutathione S-transferases. Biochem. Biophys. Res. Commun., 108: 461-467.

Tu, C.-P.D., Matsushima, A., Li, N., Rhoads, D.M., Srikumar, K., Reddy, A.P., Reddy, C.C. (1986) Immunological and sequence inter-relationships between multiple human liver and rat glutathione S-transferases. J. Biol. Chem., 261: 9540 - 9545.

Vander Jagt, D.L., Wilson, S.P. and Heidrich, J.E. (1981). Purification and bilirubin binding properties of glutathione S-transferase from human placenta. FEBS lett., 136: 319-321.

Vander Jagt, D.L., Wilson, S.P., Dean, V.L. and Simons, P.C. (1982) Bilirubin binding to rat liver ligandins (glutathione S-transferases A and B). Relationships between bilirubin binding and transferase activity. J. Biol. Chem., 257: 1997-2001.

Vander Jagt, D.L., Dean, V.L., Wilson, S.P. and Royer, R.E. (1983) Regulation of the glutathione S-transferase activity of bilirubin transport protein (ligandin) from human liver : enzymic memory and living protein - protein interactions. J. Biol. Chem., 258: 5689-5694.

Vander Jagt, D.L., Hunsaker, L.A., Garcia, K.B. and Royer, R.E. (1985) Isolation and characterization of the multiple glutathione S-transferases from human liver. J. Biol. Chem., 260: 11603-11610.

Vessey, D.A. and Zakim, D. (1981) Inhibition of glutathione S-transferase by bile acids. Biochem. J., 197: 321-325.

Vince, R., Daluge, S. and Wadd, W.B. (1971) Studies on the inhibition of glyoxylase I by S-substituted glutathiones. J. Med. Chem., 14: 402-404.

Warholm, M., Guthenberg, C., Mannervik, B., von Bahr, C. and Glaumann, H. (1980) Identification of a new glutathione S-transferase in human liver. Acta Chem. Scand., B34: 607-621.

Warholm, M., Guthenberg, C., Mannervik, B. and von Bahr, C. (1981a) Purification of a new glutathione S-transferase (transferase μ) from human liver having high activity with benzo[a]pyrene-4,5-oxide. Biochem. Biophys. Res. Commun, 98: 512-519.

Warholm, M., Guthenberg, C., Mannervik, B., Pacifica, G.M. and Rane, A. (1981b) Glutathione S-transferases in human fetal liver. Acta Chem. Scand., B35: 225-226.

Warholm, M., Guthenberg, C. and Mannervik, B. (1983) Molecular and catalytic properties of glutathione transferase from human liver: An enzyme efficiently conjugating epoxides. Biochemistry, 22: 3610-3617.

Wiegand, R.C., Shah, D.M., Mozer, T.J., Harding, E.I., Saunders, J.D.-Z., Jaworski, E.G. and Tiemeier, D.C. (1986) Messenger RNA encoding a glutathione S-transferase responsible for herbicide tolerance in maize is induced in response to safener treatment. Plant. Mol. Biol., 7: 235-243.

Wolf, C.R., Lewis, A.D., Carmichael, J., Ansell, J., Adams, D.J., Hickson, I.J., Harris, A., Balkwill, F.R., Griffin, D.B. and Hayes, J.D. (1987) Glutathione S-transferase expression in normal and tumour cells resistant to cytotoxic drugs. In: Mantle, T.J., Pickett, C.B. and Hayes, J.D. (ed.) Glutathione S-transferases and carcinogenesis. Taylor and Francis, London, pp. 199-212.

Wolkoff, A.W. (1980) The glutathione S-transferases: Their role in the transport of organic anions from blood to bile. International Rev. Physiol., 21: 151-169.

Wolkoff, A.W., Goresky, C.A., Sellin, J., Gatmaitan, Z. and Arias, I.M. (1979) Role of ligandin in transfer of bilirubin from plasma into liver. Am. J. Physiol., 236: E638-E648.

Yamada, T. and Kaplowitz, N. (1980) Binding of ethacrynic acid to hepatic glutathione S-transferases in vivo in the rat. Biochem. Pharmacol., 29: 1205-1208.

Yalcin, S., Jensson, H. and Mannervik, B. (1983) A set of inhibitions for discrimination between the basic iso-enzymes of glutathione transferase in rat liver. Biochem. Biophys. Res. Commun., 114: 829-834.

SECTION 6: PUBLICATIONS

The following publications have arisen as a result of work presented in this thesis:

1. Stockman, P.K., Beckett, G.J. and Hayes, J.D. (1985) Identification of a basic hybrid glutathione S-transferase from human liver. Glutathione S-transferase δ is composed of two distinct subunits (B_1B_2). Biochem. J., 227: 457-465.

2. Stockman, P.K. and Hayes, J.D. (1987) Identification of a Yb-containing glutathione S-transferase(GST ϕ) in human liver with an acidic pI value. In: Mantle, T.J., Pickett, C.B. and Hayes, J.D. (ed.) Glutathione S-transferases and carcinogenesis. Taylor and Francis, London, pp. 41-42.

3. Stockman, P.K., McLellan, L.I. and Hayes, J.D. (1987) Characterization of the basic glutathione S-transferase B_1 and B_2 subunits from human liver. Biochem. J., 244: 55-61.

4. Hayes, J.D., Coulthwaite, R.E., Stockman, P.K., Hussey, A.J., Mantle, T.J. and Wolf, C.R. (1987) Glutathione S-transferase subunits in the mouse and their catalytic activities towards reactive electrophiles. Arch. Toxicol. Suppl., 10: 136-146.

5. Hayes, J.D., McLellan, L.I., Stockman, P.K., Chalmers, J. and Beckett, G.J. (1987) Glutathione S-transferase in man. The relationship between rat and human enzymes. Biochem. Soc. Trans., 15: 721-725.

6. Hayes, J.D., McLellan, L.I., Stockman, P.K., Chalmers, J., Howie, A.F., Hussey, A.J. and Beckett, G.J. (1987) Human glutathione S-transferases In: 10th European Drug Metabolism Workshop. Taylor and Francis, London. In the press.

7. Hayes, J.D., McLellan, L.I., Stockman, P.K., Howie, A.F., Hussey, A.J. and Beckett, G.J. (1987) Human glutathione S-transferases: A polymorphic group of detoxification enzymes. In: Mantle, T.J., Pickett, C.B. and Hayes, J.D. (ed.) Glutathione S-transferases and carcinogenesis. Taylor and Francis, London, pp. 3-18.

8. Hussey, A.J., Stockman, P.K., Beckett, G.J. and Hayes, J.D. (1986) Variations in the glutathione S-transferase subunits expressed in human livers. Biochim. Biophys. Acta, 874: 1-12.

Biochem. J. (1985) **227**, 457–465
Printed in Great Britain

Identification of a basic hybrid glutathione *S*-transferase from human liver

Glutathione *S*-transferase δ is composed of two distinct subunits (B_1 and B_2)

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(*Received 21 September 1984/Accepted 11 December 1984*)

The purification of a hybrid glutathione *S*-transferase (B_1B_2) from human liver is described. This enzyme has an isoelectric point of 8.75 and the B_1 and B_2 subunits are distinguishable immunologically and are ionically distinct. Hybridization experiments demonstrated that B_1B_1 and B_2B_2 could be resolved by CM-cellulose chromatography and have pI values of 8.9 and 8.4 respectively. Transferase B_1B_2, and the two homodimers from which it is formed, are electrophoretically and immunochemically distinct from the neutral enzyme (transferase μ) and two acidic enzymes (transferases ρ and λ). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis demonstrated that B_1 and B_2 both have an M_r of 26000, whereas, in contrast, transferase μ comprises subunits of M_r 27000 and transferases ρ and λ both comprise subunits of M_r 24500. Antisera raised against B_1 or B_2 monomers did not cross-react with the neutral or acidic glutathione *S*-transferases. The identity of transferase B_1B_2 with glutathione *S*-transferase δ prepared by the method of Kamisaka, Habig, Ketley, Arias & Jakoby [(1975) *Eur. J. Biochem.* **60**, 153–161] has been demonstrated, as well as its relationship to other previously described transferases.

The first step in the detoxification of a wide range of electrophilic compounds is conjugation with GSH (Boyland & Chasseaud, 1969). This reaction is catalysed by glutathione *S*-transferase (EC 2.5.1.18), an enzyme that occurs in a variety of structural forms.

Extensive information exists about the genetic relationships between the transferases in the rat, but relatively little is known about the human enzymes. Nonetheless, it is widely accepted that the multiple transferase forms which exist in man and the rat arise by entirely different mechanisms (Jakoby & Habig, 1980).

At least 12 glutathione *S*-transferases have been described in rat liver (Gillham, 1973; Hayes & Chalmers, 1983; Hayes, 1984; Meyer *et al.*, 1984). These rat enzymes each comprise two subunits and are coded for by a limited number of genes. Hetero- and homo-dimers exist, and the large number of forms that have been isolated arise as the result of subunit hybridization (Hayes *et al.*, 1981; Beale *et al.*, 1982, 1983; Mannervik & Jensson, 1982; Hayes, 1983, 1984).

In contrast with the rat enzymes, the human transferases exhibit very limited substrate specificities and are thought to represent merely charge isomers arising from deamidation (Kamisaka *et al.*, 1975). Proteolysis has also been suggested as the mechanism responsible for the generation of the multiple forms in man (Grover, 1982). The fact that, to date, no hybrid forms have been described in man has only served to support the hypothesis that the human and rat enzymes are fundamentally different. It is currently believed that all human enzymes are homodimers (for a review see Mannervik, 1985).

The human transferases can be divided according to their charge into three distinct groups; the basic, the neutral and the acidic enzymes (Warholm *et al.*, 1981). Although these three groups are widely thought to be genetically distinct, it has been reported that the basic and acidic groups are immunologically inter-related (Awasthi *et al.*, 1980).

Abbreviations used: GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; SP-Sephadex, sulphopropyl-Sephadex; SDS, sodium dodecyl sulphate.

The nomenclature used to describe the basic transferases is based on the purification scheme devised by Kamisaka *et al.* (1975). These workers originally isolated and characterized five basic transferases, α, β, γ, δ and ε, from human liver. Kamisaka *et al.* (1975) applied human liver extract to DEAE-cellulose, and the activity that was eluted in the 'flow-through' fractions was resolved by CM-cellulose chromatography. Transferases α, β and γ did not bind the CM-cellulose (these were resolved by hydroxyapatite and column isoelectric focusing), whereas transferases δ and ε were eluted sequentially from the cation-exchanger by a KCl gradient. Transferases α–γ had pI values of 7.8–8.55, whereas transferases δ and ε had pI values of 8.75 and 8.8 respectively. The physicochemical and immunochemical properties of these enzymes led Kamisaka *et al.* (1975) to suggest that transferases α–ε were formed as the result of deamidation of a single gene product. The antisera that Kamisaka *et al.* (1975) raised against either the α-, β-, γ-, δ- and ε-forms showed equal cross-reactivity with each of the basic enzymes.

Here we present evidence that deamidation is not responsible for the generation of all the cationic glutathione *S*-transferases isolated from human liver. The existence of two immunologically and ionically distinct basic glutathione *S*-transferase subunits (B_1 and B_2) is demonstrated. These two monomers can hybridize, and the hybrid enzyme is present in all liver specimens investigated. The purification scheme of Kamisaka *et al.* (1975), which is regarded as the definitive method for the isolation of basic transferases, has been employed to identify the hybrid enzyme.

Experimental

Materials

Chemicals were all of analytical grade and readily available commercially.

Normal rabbit serum and donkey anti-rabbit serum were obtained from the Scottish Antibody Production Unit, Carluke, Lanarkshire, Scotland, U.K.

Liver

Human liver was obtained 24h *post mortem* from individuals who had died from a disease that had not involved the liver. The livers were stored at −70°C until required.

Analytical methods

Glutathione S-transferase enzymic assays. Transferase activity, with 2mM-GSH and 1mM-CDNB as substrates, was measured as described previously (Hayes & Clarkson, 1982).

Transferase activity, with 0.05mM-*trans*-4-phenylbut-3-en-2-one and 0.025mM-GSH as substrates, was measured at 37°C as described previously (Habig *et al.*, 1974).

Radioimmunoassay for glutathione S-transferase. Radioimmunoassay of various glutathione *S*-transferases was carried out as described previously (Beckett & Hayes, 1984), by using antisera raised to transferase B_1B_1 and to B_2B_2 [previously referred to as 'Basic' and 'N/A2b' respectively (Hayes *et al.*, 1983)]. The relative cross-reactivity was defined as that quantity of transferase that was required to produce 50% displacement of bound ligand when compared with the most immunoreactive protein.

SDS/polyacrylamide-gel electrophoresis. Electrophoresis was performed as described by Laemmli (1970).

Isoelectric focusing. Analytical isoelectric focusing in thin-layer 5% (w/v) polyacrylamide gel was performed with an LKB Multiphor apparatus as described by the manufacturer (LKB-Produkter, Bromma, Sweden). Both broad-range (pH 3.5–9.5) and narrow-range (pH 7.5–10.0) gels were used to determine isoelectric points. Protein standards with basic isoelectric points were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Enzyme purification

(*a*) *Purification of B_1B_2 protein.* Human liver cytosol was prepared and the non-diffusible material (355ml, about 8g of protein) applied to a column (3.2cm × 78.2cm) of DEAE-Sephadex eluted as described previously (Hayes *et al.*, 1983). The glutathione *S*-transferases that were retained by the DEAE-Sephadex column were eluted as two peaks of activity by a 0–250mM-NaCl gradient. The first peak, which was eluted at an Na^+ concentration of 61mM, was pooled and applied to an *S*-hexylglutathione affinity column (Mannervik & Guthenberg, 1981). The material eluted by 5mM-*S*-hexylglutathione was combined and dialysed against two changes, each of 1 litre, of 5mM-Tris/HCl, pH 8.0, for 12h. The non-diffusible material was concentrated to 5–10ml by ultrafiltration and eluted from a chromatofocusing column (1.6cm × 32.0cm) by a pH 9.0–6.8 gradient.

Transferases B_1B_1 and B_2B_2 were prepared from human liver as described previously (Hayes *et al.*, 1983).

(*b*) *Purification of glutathione S-transferase μ.* This was purified by using the method described above for B_1B_2 protein. Transferase μ was resolved from B_1B_2 protein by the final chromatofocusing step.

(*c*) *Purification of glutathione S-transferases ρ and λ.* These enzymes were isolated by using a strategy similar to that used by Marcus *et al.* (1978). Erythrocyte or lung extracts were applied to

a SP-Sephadex column (4.4cm × 86.0cm) equilibrated with 10mM-sodium phosphate, pH 6.5. The material that was eluted in the 'flow-through' fractions was subjected to *S*-hexylglutathione affinity chromatography and was finally purified by elution from PBE 94 chromatofocusing columns (1.6cm × 35.0cm) with a pH 6.0–4.0 gradient.

Reversible dissociation and separation of the products obtained from glutathione S-transferase B_1B_2 and glutathione S-transferase μ

The subunits from B_1B_2 protein were dissociated by 6M-guanidinium chloride denaturation, and the products formed after re-association were resolved by DEAE-Sephadex chromatography (Kitahara & Sato, 1981). The DEAE-Sephadex column (2.2cm × 21.0cm) was equilibrated with 20mM-Tris/HCl, pH 7.8, and the proteins were eluted with a 0–250mM-NaCl gradient.

Glutathione *S*-transferase μ was also subjected to 6M-guanidinium chloride, and after extensive dialysis the renatured products were applied to DEAE-Sephadex.

The various products of reversible dissociation of glutathione *S*-transferase B_1B_2, which had been separated by using DEAE-Sephadex chromatography, were subjected to CM-cellulose chromatography at pH 6.7 to facilitate their identification.

Preparation and identification of glutathione S-transferase δ by using the 'standard' purification scheme

The basic glutathione *S*-transferases were prepared as described by Kamisaka *et al.* (1975), since this method represents the basis of the currently used nomenclature describing these enzymes.

About 306g of frozen liver was allowed to thaw at 20°C before being cut into small pieces and blended in 1.2 litres of ice-cold distilled water. All subsequent steps were carried out at 4°C. The extract was centrifuged at 10000***g*** for 2h, and lipid was removed from the supernatant by filtration through a plug of glass-wool. This preparation was applied to a column (3.2cm × 85.0cm) of DEAE-cellulose equilibrated with 10mM-Tris/HCl, pH 8.0. The column was washed with the same buffer until free of transferase activity. These 'flow-through' fractions from DEAE-cellulose were combined (821ml) and concentrated by $(NH_4)_2SO_4$ fractionation. The protein that was precipitated at 95% $(NH_4)_2SO_4$ saturation was dissolved in approx. 120ml of 10mM-potassium phosphate, pH 6.7, and dialysed for 20h against three changes, each of 2 litres, of the same buffer. The non-diffusible material (164ml, about 0.3g of protein) was applied to a column (3.2cm × 56.0cm) of CM-cellulose that had been equilibrated with 10mM-sodium phosphate, pH 6.7. The column was washed with approx. 600ml of the running buffer before a 0–150mM-KCl gradient was applied.

Results

Purification of the hybrid transferase, B_1B_2 protein

The purification scheme for transferase B_1B_2 employed DEAE-Sephadex chromatography, *S*-hexylglutathione affinity chromatography and, finally, chromatofocusing over the pH range 9.0–6.8 as described above. Chromatofocusing resulted in the resolution of a major transferase peak that was eluted at pH 8.3 and is referred to as 'B_1B_2' (Fig. 1). Analysis of B_1B_2 protein by SDS/polyacrylamide-gel electrophoresis showed that it migrated as a single band that ran between the Ya

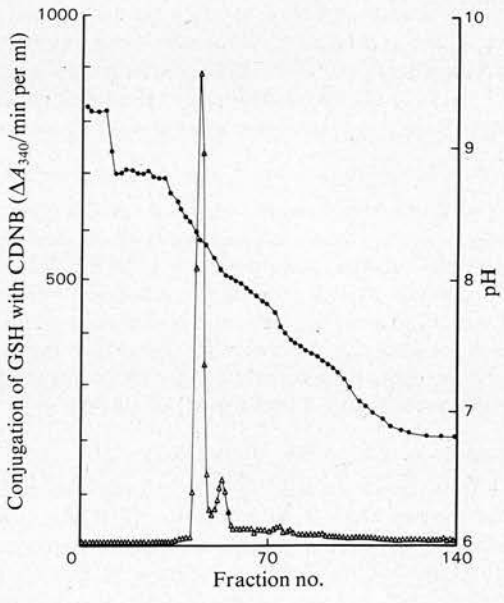

Fig. 1. *Chromatofocusing of glutathione S-transferase B_1B_2*

The glutathione *S*-transferases that were retained by DEAE-Sephadex and eluted in the first peak on the salt gradient (at [Na^+] 44–83mM) were purified by *S*-hexylglutathione affinity chromatography. The resulting material (about 7mg of protein) was dialysed against 2 × 1 litre of 5mM-Tris/HCl, pH 8.0, and, after concentration to about 5ml, was applied to a column (1.6cm × 32.0cm) of chromatofocusing gel PBE 94. This column was equilibrated with 25mM-ethanolamine/acetate, pH 9.4, and eluted (27ml/h) with Polybuffer 96 adjusted with acetic acid to pH 6.8. Fractions of volume 6.6ml were collected. The pH (●) and transferase activity with CDNB (△) in the eluate were determined. This elution profile was obtained from a liver whose phenotype was different from that of the liver used for Fig. 2; it did not express transferase μ.

and Yb monomers from rat liver. Both subunits have an apparent M_r of 26000. Isoelectric focusing also demonstrated the purity of the B_1B_2 preparation and revealed that it had a pI of 8.75 when compared with pI markers trypsinogen (pI9.30) and lentil lectin, which has pI8.65 (basic band), 8.45 (middle band) and 8.15 (acidic band).

Purification of glutathione S-transferase μ

Transferase μ possessed elution characteristics from DEAE-Sephadex similar to those of B_1B_2, but could be resolved from the latter by chromatofocusing on PBE 94. The purification strategy used for B_1B_2 was therefore also employed to isolate transferase μ. In contrast with B_1B_2, transferase μ was eluted from the final chromatofocusing column at pH 7.7 (Fig. 2). This enzyme was identified as transferase μ because it exhibited a high specific activity for *trans*-4-phenylbut-3-en-2-one. Furthermore, transferase μ could be distinguished from B_1B_2, as it had a pI value of 6.1 and an apparent subunit M_r of 27000. Both isoelectric focusing and SDS/polyacrylamide-gel electrophoresis demonstrated that transferase μ was homogeneous.

Purification of glutathione S-transferases ρ and λ

These enzymes were purified as described above. Transferases ρ and λ were both eluted from chromatofocusing columns at pH 4.8. During SDS/polyacrylamide-gel electrophoresis, transferases ρ and λ co-migrated and possessed a slightly faster electrophoretic mobility than did the Ya monomer from rat liver; the monomers comprising transferases ρ and λ had an M_r of 24500.

Immunoreactivity of the transferases

The antisera raised against transferase B_1B_1 cross-reacted with B_1B_2 but not with B_2B_2. Conversely, antisera raised against B_2B_2 cross-reacted with B_1B_2 but not with transferase B_1B_1. These antisera did not cross-react with the neutral enzyme, glutathione S-transferase μ, nor with the acidic enzymes, transferases λ and ρ (Table 1).

Reversible dissociation of transferase B_1B_2

After reversible dissociation of B_1B_2, three peaks of CDNB–GSH-conjugating activity were

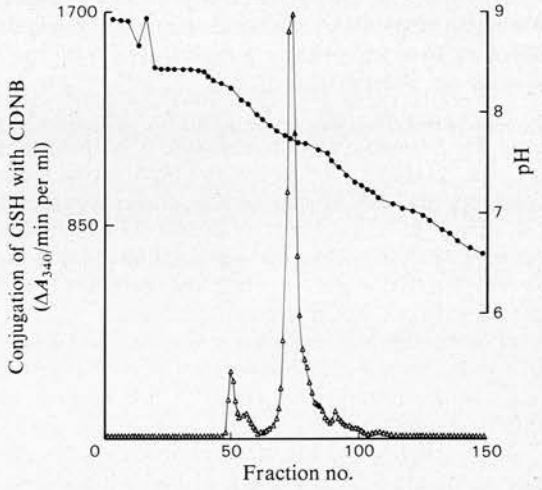

Fig. 2. *Chromatofocusing of glutathione S-transferase μ*
The glutathione S-transferases that were retained by DEAE-Sephadex and eluted in the first peak on the salt gradient (at [Na^+] 44–83 mM) were purified by S-hexylglutathione affinity chromatography. The resulting material (about 10 mg of protein) was dialysed against 2 × 1 litre of 25 mM-ethanolamine/acetate, pH 9.0, and applied to a column (1.6 cm × 45.0 cm) of chromatofocusing gel PBE 94. This column was eluted (18 ml/h) with Polybuffer 96 adjusted with acetic acid to pH 6.5, and 5.8 ml fractions were collected. The pH (●) and transferase activity with CDNB (△) in the eluate were determined. This elution profile was obtained from a liver whose phenotype was different from that of the liver used for Fig. 1; it contained little transferase B_1B_2.

Table 1. *Immunochemical, chromatographic and electrophoretic properties of the glutathione S-transferases in man*
For full experimental details see the text. Abbreviation: N.D., not determined.

Glutathione S-transferase	Relative cross-reactivity of antiserum (%)		pH of its elution from the chromatofocusing column	pI value	$10^{-3} \times M_r$
	Anti-B_1	Anti-B_2			
B_1B_1	100	0.2	9.0	8.9	26.0
B_1B_2	55	58	8.3	8.75	26.0
B_2B_2	0.3	100	N.D.	8.4	26.0
μ	<0.1	<0.1	7.7	6.1	27.0
ρ	<0.1	<0.1	4.8	4.9*	24.5
λ	<0.1	<0.1	4.8	N.D.	24.5

* pI value from Marcus *et al.* (1978).

resolved (Fig. 3*a*). The first peak was not retained by the column; the second and third peaks were eluted by the salt gradient at Na^+ concentrations of 48mM and 94mM respectively. Native glutathione *S*-transferase B_1B_2, which had not been subjected to guanidinium chloride treatment, was eluted in parallel from an identical DEAE-Sephadex column and served as a control (Fig. 3*b*).

Antisera raised against transferase B_2B_2 showed high cross-reactivity with the second and third peaks of activity, but not with the first peak.

Reversible dissociation of transferase μ

When glutathione *S*-transferase μ that had been subjected to reversible dissociation was applied to DEAE-Sephadex, it was eluted on the salt gradient as a single peak of activity at an Na^+ concentration of 49mM.

Analysis of the products of reversible dissociation of transferase B_1B_2

The three products of reversible dissociation of B_1B_2 that were separated on DEAE-Sephadex were individually subjected to isoelectric focusing and CM-cellulose chromatography to help in their identification.

Transferases B_1B_1, B_1B_2 and B_2B_2 were separately subjected to CM-cellulose chromatography under identical conditions. Transferase B_2B_2 was eluted in the 'flow-through' fractions, whereas B_1B_1 was eluted as a single peak on the salt gradient at 45mM. However, transferase B_1B_2 was eluted as two peaks on the salt gradient at Na^+ concentrations 25mM and 36mM (Fig. 4*b*). Each of these, when separately re-applied to identical columns after storage (4°C for 30 days), was co-eluted as a single peak at a position corresponding to the form of the enzyme that was originally eluted at the higher Na^+ concentration (Figs. 4*d* and 4*e*).

Portions (1–10μg) of transferases B_1B_1, B_1B_2 and B_2B_2 were subjected to isoelectric focusing and resulted in the resolution of major bands of protein with pI values of 8.9, 8.75 and 8.4 respectively.

Identification of the hybrid enzyme (B_1B_2) as a member of the transferase α–ε family

The nomenclature used to describe the basic transferases is based on their elution order from CM-cellulose (Kamisaka *et al.*, 1975).

To enable the hybrid transferase to be identified, a liver of the appropriate phenotype was subjected to the purification scheme of Kamisaka *et al.* (1975). The liver chosen had previously been shown to contain large amounts of B_1B_1 and B_1B_2 protein, but possessed little if any B_2B_2 protein or

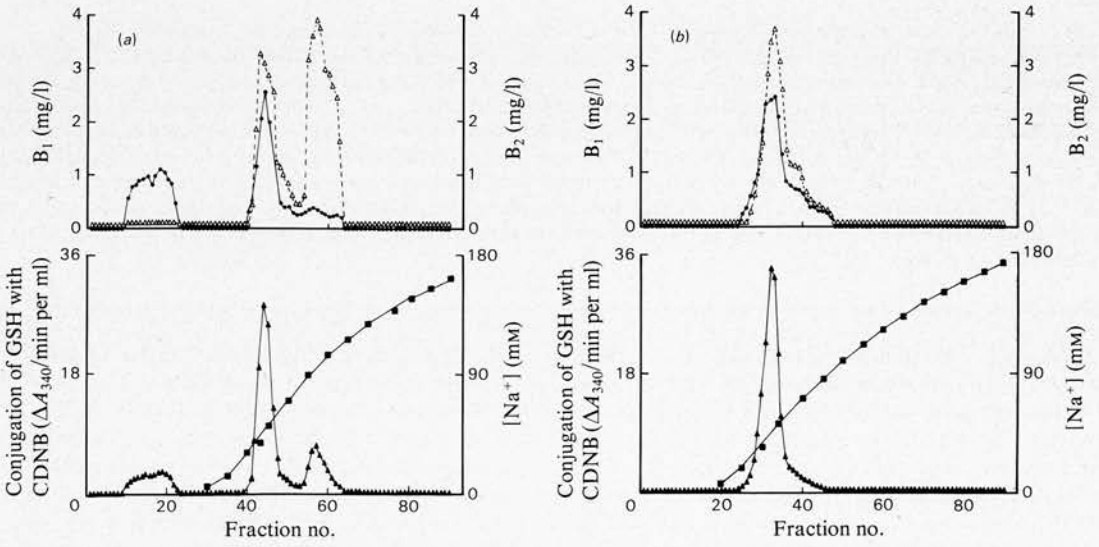

Fig. 3. *Separation of the products of reversible dissociation of transferase B_1B_2*

(*a*) Transferase B_1B_2 (0.5mg of protein) was incubated with 6M-guanidinium chloride, followed by dialysis against 20mM-Tris/HCl, pH7.8. The non-diffusible material was applied to a column (2.2cm × 21cm) of DEAE-Sephadex that had been equilibrated with 20mM-Tris/HCl, pH7.8, and a gradient of 0–250mM-NaCl was used to develop the column. (*b*) A portion of transferase B_1B_2 that had not been incubated with 6M-guanidinium chloride was also applied to an identical DEAE-Sephadex column. The Na^+ concentration (■), transferase activity with CDNB (▲) and cross-reactivity with anti-B_1B_1 (●) and anti-B_2B_2 (△) sera were determined.

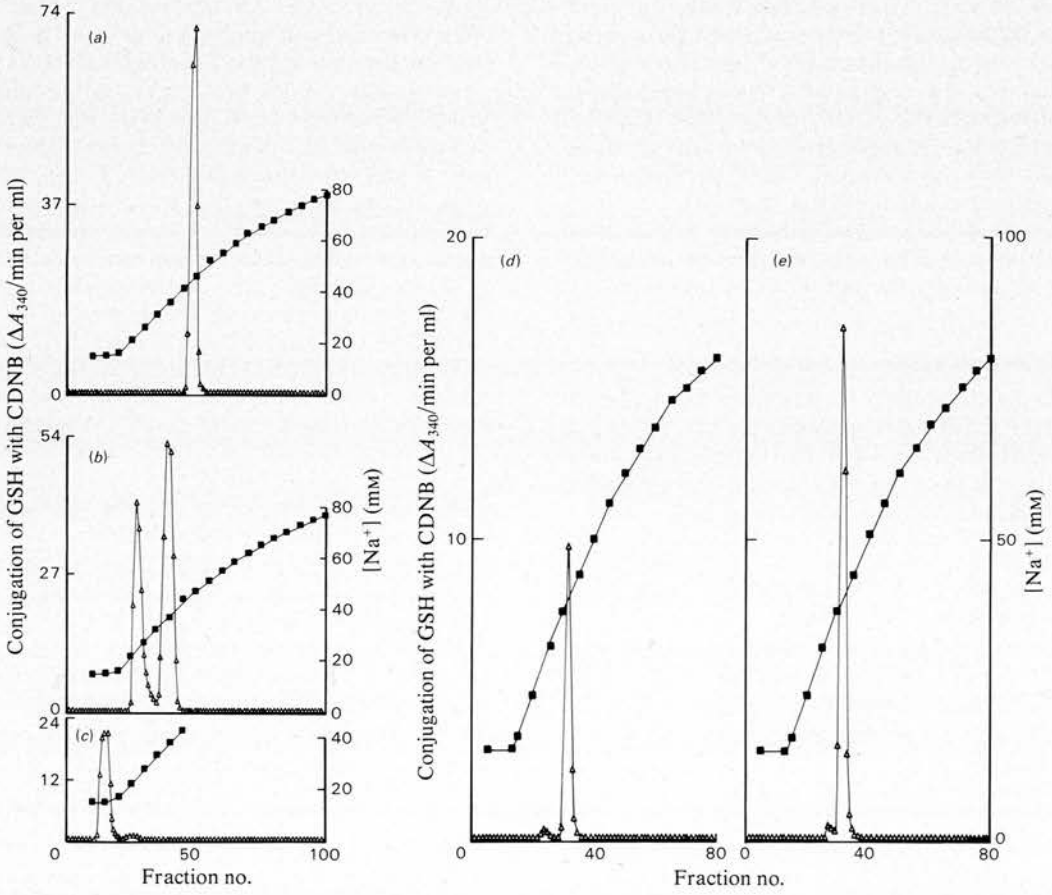

Fig. 4. *CM-cellulose chromatography of the re-associated products obtained from transferase B_1B_2*

Transferases B_1B_1, B_1B_2 and B_2B_2, which were obtained by reversible dissociation of transferase B_1B_2, were resolved by DEAE-Sephadex chromatography as described in the text. Portions (25–60μg of protein) of these enzymes were dialysed against 2 × 1 litre of 10mM-sodium phosphate, pH6.7, before being applied to identical columns (2.2cm × 24.0cm) equilibrated with the same buffer. A 0–100mM-NaCl gradient was used to develop these columns. Fractions (4.0ml) were collected and the Na^+ concentration (■) and transferase activity with CDNB (△) were measured. (*a*) B_1B_1; (*b*) B_1B_2; (*c*) B_2B_2. This procedure resulted in the resolution of two peaks of enzyme activity from transferase B_1B_2. Each of these was resubjected to chromatography and analysed by using the conditions described. Fractions (3.8ml) were collected. (*d*) First peak from profile shown in (*b*); (*e*) second peak from profile shown in (*b*).

transferase μ. The remaining portions of this liver, used to identify which transferase corresponded to B_1B_2 protein, were subjected to DEAE-cellulose as described by Kamisaka *et al.* (1975) before the CM-cellulose step (see above). The material that was not retained by DEAE-cellulose, and was enriched for basic transferases, was concentrated by $(NH_4)_2SO_4$ precipitation before being applied to the CM-cellulose column. Examination of the eluate from this cation-exchanger revealed a broad peak of enzyme activity in the 'flow-through' fractions and two major peaks of activity that were resolved by the salt gradient (Fig. 5). The two major peaks of activity resolved by the gradient were identified, according to their order of elution, as transferases δ and ε respectively.

Antisera raised to transferase B_1B_1 cross-reacted with each of the peaks of activity from CM-cellulose resolved by the salt gradient. In contrast, the B_2B_2 antisera only cross-reacted appreciably with the first peak resolved on the salt gradient. This peak corresponded to transferase δ described by Kamisaka *et al.* (1975) (Fig. 5). The anti-B_1 and anti-B_2 sera reacted equally with the transferase δ-containing peak from CM-cellulose, whereas the anti-B_1 serum showed approx. 50-fold greater reactivity than the anti-B_2 serum for the transferase ε-containing peak.

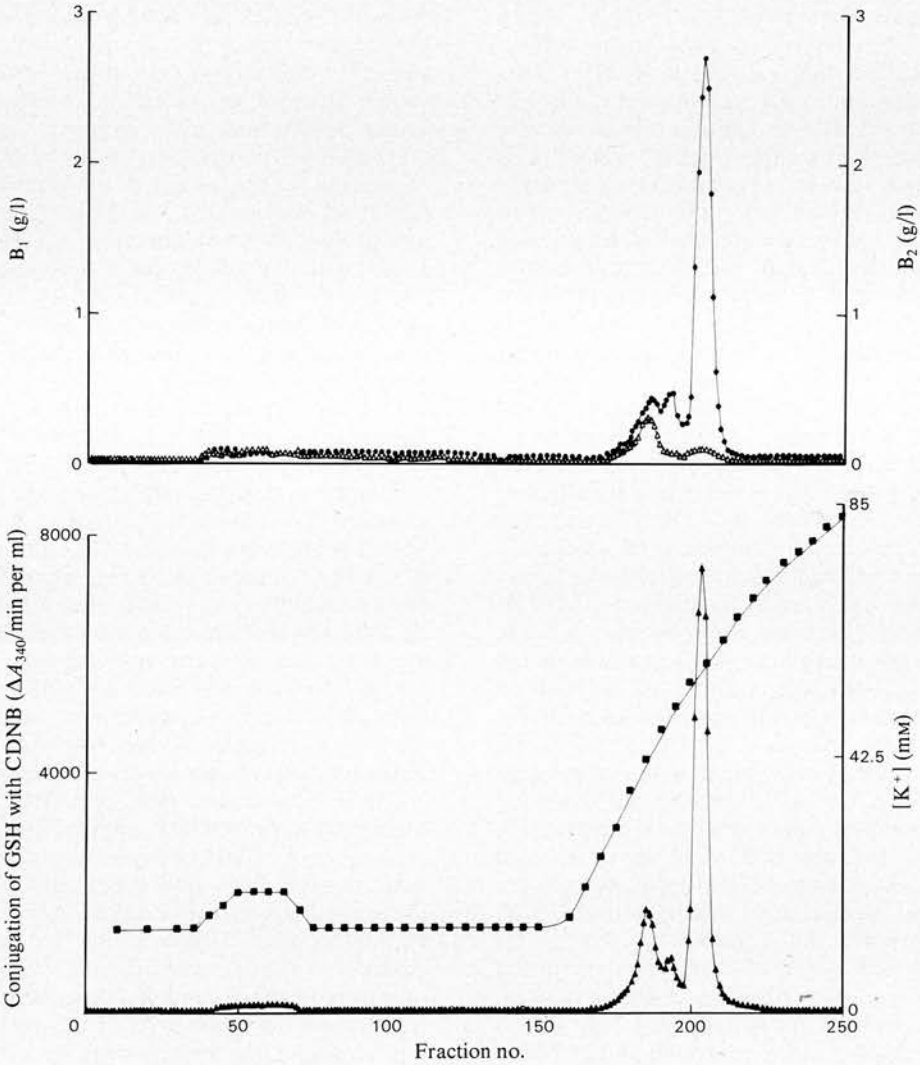

Fig. 5. *Elution pattern from CM-cellulose of human liver extract*

The 'flow-through' fractions obtained when human liver extract was applied to a 3.2cm × 85.0cm DEAE-cellulose column were concentrated by $(NH_4)_2SO_4$ precipitation and dialysed against 10mM-potassium phosphate, pH6.7. The non-diffusible material (0.3g of protein) was applied to a column (3.2cm × 56.0cm) of CM-cellulose that had been equilibrated with 10mM-potassium phosphate, pH6.7. After a washing with approx. 600ml of the running buffer, a 0–150mM-KCl gradient was applied. Fractions (8.6ml) were collected and the Na^+ concentration (■), transferase activity with CDNB (▲) and cross-reactivity with anti-B_1B_1 (●) and anti-B_2B_2 (△) sera were determined.

Discussion

The glutathione *S*-transferases are an important group of detoxification enzymes. Several forms have been isolated from human tissues; some of these are absent from certain individuals. However, the genetic relationship between the various transferases found in man is poorly understood.

Five basic glutathione *S*-transferases (pI > 7.5), called α–ε, have been isolated from human liver. The similar catalytic and physicochemical properties of these enzymes led Kamisaka *et al.* (1975) to propose they are coded for by a single gene and are produced by deamidation. However, in the present paper we have demonstrated the existence of two distinct basic transferase subunits, which can form hetero- and homo-dimers. These two subunits are referred to as 'B_1' and 'B_2'.

We have found that the basic transferases are best resolved with DEAE-Sephadex equilibrated

at pH 7.8. Under these conditions the B_1B_1 homodimer is eluted in the 'flow-through' fractions from DEAE-Sephadex, whereas the B_1B_2 and B_2B_2 forms are sequentially eluted from the exchanger by a salt gradient. The recognition that transferase B_1B_2 comprises two non-identical subunits is based on the results of hybridization experiments and on its cross-reactivity with antisera raised against the two parental homodimers, transferase B_1B_1 and transferase B_2B_2 [previously referred to as 'Basic' and 'N/A2b' respectively (Hayes *et al.*, 1983; Beckett & Hayes, 1984)]. The existence of a hybrid human transferase has not been previously reported.

To establish the relationship of the hybrid enzyme with the previously described basic transferases (α–ε), each of the three peaks of re-associated enzyme obtained from the hybrid transferase, which were resolved by DEAE-Sephadex chromatography, were subjected to isoelectric focusing and CM-cellulose chromatography; these were the techniques used by Kamisaka *et al.* (1975) to discriminate between transferases α–ε. The B_2B_2 homodimer had a pI of 8.4 and was eluted from the CM-cellulose column at a position equivalent to that originally reported for α, β or γ. The B_1B_1 homodimer had a pI of 8.9 and was eluted from the CM-cellulose column at a position equivalent to ε. The B_1B_2 heterodimer possessed the same isoelectric point (pI 8.75) as transferase δ. However, transferase B_1B_2 was eluted as two peaks of activity from the CM-cellulose column, both of which cross-reacted equally with the B_1B_1 and B_2B_2 antisera, and appeared to interconvert when re-applied to the column. Interconverting forms of δ were described by Kamisaka *et al.* (1975), but were not further investigated.

Since these hybridization results suggest that transferase δ could be the B_1B_2 heterodimer, transferase δ was prepared by using the original method of Kamisaka *et al.* (1975). The CM-cellulose column profile of human basic transferases obtained by the method of Kamisaka *et al.* (1975) resulted in the clear resolution of transferases δ and ε. The cross-reactivity of the fractions with the B_1B_1 and B_2B_2 antisera demonstrated that transferase δ, the first peak of enzyme activity resolved by the KCl gradient, was the hybrid enzyme (B_1B_2). The second major peak of activity resolved by the KCl gradient, ε, only cross-reacted with the B_1B_1 antisera, indicating that it was a B_1B_1 homodimer. A minor peak of activity was eluted between δ and ε. This peak, which was not resolved by Kamisaka *et al.* (1975), only cross-reacted with the B_1B_1 antisera.

Thus, although we have clearly demonstrated that the hybrid enzyme, B_1B_2, and the B_1B_1 homodimer are equivalent to transferases δ and ε respectively, it is less clear which transferase(s) the B_2B_2 homodimer represents. The electrophoretic properties of B_2B_2 show it comprises subunits of apparent M_r 26000 that are distinct from those found in the neutral and acidic enzymes, and has a pI value of 8.4. The isoelectric point of B_2B_2 lies between the values reported by Kamisaka *et al.* (1975) for transferases β and γ (8.25 and 8.55 respectively). However, in view of the fact that Kamisaka *et al.* (1975) could only resolve native cytosol into three peaks of activity by polyacrylamide-gel electrofocusing, it is possible that transferases α, β and γ arise by autoxidation of a single gene product during the purification scheme. An analogous situation may exist in the rat for the Yb_1 monomer (Hayes & Clarkson, 1982; Friedberg *et al.*, 1983).

The B_1 and B_2 subunits are not catalytically identical. We have found that the B_1B_1 protein possesses apparent K_m values for GSH and CDNB of 0.4 and 1.0 mM respectively, whereas the B_2B_2 protein exhibits values of 0.33 and 0.43 mM. The K_m values of the heterodimer for GSH and CDNB are 0.35 and 0.76 mM respectively. There are several reports in the literature where basic fractions have been combined and treated experimentally as a single enzyme (Awasthi *et al.*, 1980; Pattinson, 1981; Sherman *et al.*, 1983; Warholm *et al.*, 1983; Dao *et al.*, 1984). Since the B_1 and B_2 monomers appear to be catalytically distinct, a re-evaluation of certain catalytic data describing the basic transferases would appear prudent. Indeed, the preliminary work of Koskelo & Icén (1984) can be interpreted as demonstrating that very marked differences exist between the inhibitory effects of bilirubin on the B_1 and B_2 monomers.

Although this is the first formal identification of two ionically and immunologically distinct basic glutathione *S*-transferase subunits in man, Board (1981) postulated, from analysis of zymogram patterns, the existence of two electrophoretically distinct human cationic transferase subunits, which could form hybrids.

Board (1981) demonstrated that, when liver cytosol was subjected to starch-gel electrophoresis at pH 8.6, multiple bands of glutathione *S*-transferase activity were revealed, with CDNB as substrate, that migrated towards both anode and cathode. The strongly staining anodal components were proposed to be the product of one locus, GST_1. The GST_1 zone was not always present in tissue and its frequency of occurrence is consistent with it representing the neutral transferase μ. The cathodal components are the products of the GST_2 locus. The charge, frequency of occurrence and multiple band patterns of this component are consistent with it representing the basic group of enzymes. A weakly staining very-fast-migrating

anodal component is the acidic glutathione *S*-transferase product of the GST_3 locus. This component may originate from erythrocyte contamination of liver tissue. Both the GST_1 and GST_2 components exhibit triplet patterns that are characteristic of those obtained from heterozygotes for a polymorphic dimeric protein (Board, 1981). The populations sampled appeared to achieve Hardy–Weinberg equilibrium if it was assumed that two alleles were present at the GST_2 locus, one allele at the GST_3 locus and three alleles at the GST_1 locus.

Recent work has cast doubt on the interpretation of the zymogram data by Board (1981) for the GST_2 locus. Strange *et al.* (1984), using the same technique, reasoned that the various zones of cationic transferase activity resolved by electrophoresis arose by a post-translational modification. It is, therefore, not yet clear how useful zymogram analysis is in studying the variability of cationic transferases in man.

Our data show we have isolated the two allelic products of the GST_2 locus. As we have demonstrated, the allelic products of the GST_2 locus are immunologically distinct and have separate isoelectric points. It is apparent that the polymorphism of the basic transferases in man will be best studied by using isoelectric focusing and 'Western blotting' techniques (Towbin *et al.*, 1979). This approach will be valuable in assessing whether multiple forms of B_2B_2 exist (i.e. α, β and γ).

A wide variety of reactive and toxic metabolites are eliminated from the body by the actions of glutathione *S*-transferases. Since individuals lacking certain isoenzymes have been described in this and other (Board, 1981; Warholm *et al.*, 1983) papers, it is important to establish their role in relation to the susceptibility of certain individuals to the toxic effects of particular electrophiles. The discovery of a hybrid enzyme is fundamental to our understanding of the structure–function relationship of these enzymes.

We thank Professor L. G. Whitby for critically reading this manuscript. We also thank Mr. J. Chalmers for help in purification of acidic transferases and Miss A. J. Hussey for her technical assistance in preparing the radioimmunoassay reagents. The secretarial assistance of Mrs. E. Ward is gratefully acknowledged. This work was supported by a Medical Research Council Project Grant (G8126 392 SB) awarded to J. D. H.

References

Awasthi, Y. C., Dao, D. D. & Saneto, R. P. (1980) *Biochem. J.* **191**, 1–10

Beale, D., Ketterer, B., Carne, T., Meyer, D. J. & Taylor, J. B. (1982) *Eur. J. Biochem.* **126**, 459–463

Beale, D., Meyer, D. J., Taylor, J. B. & Ketterer, B. (1983) *Eur. J. Biochem.* **137**, 125–129

Beckett, G. J. & Hayes, J. D. (1984) *Clin. Chim. Acta* **141**, 267–273

Board, P. G. (1981) *Am. J. Hum. Genet.* **33**, 36–43

Boyland, E. & Chasseaud, L. F. (1969) *Adv. Enzymol.* **32**, 173–219

Dao, D. D., Partridge, C. A., Kurosky, A. & Awasthi, Y. C. (1984) *Biochem. J.* **221**, 33–41

Friedberg, T., Milbert, U., Bentley, P., Guenthner, T. M. & Oesch, F. (1983) *Biochem. J.* **215**, 617–625

Gillham, B. (1973) *Biochem. J.* **135**, 797–803

Grover, P. L. (1982) *Biochem. Soc. Trans.* **10**, 80–82

Habig, W. H., Pabst, M. G. & Jakoby, W. B. (1974) *J. Biol. Chem.* **249**, 7130–7139

Hayes, J. D. (1983) *Biochem. J.* **213**, 625–633

Hayes, J. D. (1984) *Biochem. J.* **224**, 839–852

Hayes, J. D. & Chalmers, J. (1983) *Biochem. J.* **215**, 581–588

Hayes, J. D. & Clarkson, G. H. D. (1982) *Biochem. J.* **207**, 459–470

Hayes, J. D., Strange, R. C. & Percy-Robb, I. W. (1981) *Biochem. J.* **197**, 491–502

Hayes, J. D., Gilligan, D., Chapman, B. J. & Beckett, G. J. (1983) *Clin. Chim. Acta* **134**, 107–121

Jakoby, W. B. & Habig, W. H. (1980) in *Enzymatic Basis of Detoxification* (Jakoby, W. B., ed), pp. 63–95, Academic Press, New York

Kamisaka, K., Habig, W. H., Ketley, J. N., Arias, I. M. & Jakoby, W. B. (1975) *Eur. J. Biochem.* **60**, 153–161

Kitahara, A. & Sato, K. (1981) *Biochem. Biophys. Res. Commun.* **103**, 943–950

Koskelo, K. & Icén, A. (1984) *Scand. J. Clin. Lab. Invest.* **44**, 159–162

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Mannervik, B. (1985) *Adv. Enzymol. Relat. Areas Mol. Biol.* **57**, 357–417

Mannervik, B. & Guthenberg, C. (1981) *Methods Enzymol.* **77**, 231–235

Mannervik, B. & Jensson, H. (1982) *J. Biol. Chem.* **257**, 9909–9912

Marcus, C. J., Habig, W. H. & Jakoby, W. B. (1978) *Arch. Biochem. Biophys.* **188**, 287–293

Meyer, D. J., Christodoulides, L. G., Tan, K. H. & Ketterer, B. (1984) *FEBS Lett.* **173**, 327–330

Pattinson, N. R. (1981) *Biochem. Biophys. Res. Commun.* **102**, 403–410

Sherman, M., Titmuss, S. & Kirsch, R. E. (1983) *Biochem. Int.* **6**, 109–118

Strange, R. C., Faulder, C. G., David, B. A., Hume, R., Brown, J. A. H., Cotton, W. & Hopkinson, D. A. (1984) *Ann. Hum. Genet.* **48**, 11–20

Towbin, H., Staehlin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354

Warholm, M., Guthenberg, C. & Mannervik, B. (1981) *Biochem. Biophys. Res. Commun.* **98**, 512–519

Warholm, M., Guthenberg, C. & Mannervik, B. (1983) *Biochemistry* **22**, 3610–3617

IDENTIFICATION OF A Yb-CONTAINING GLUTATHIONE S-TRANSFERASE (GST ϕ) IN HUMAN LIVER WITH AN ACIDIC pI VALUE

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Glutathione S-transferase ϕ was isolated from human liver cytosol by DEAE-cellulose, S-hexylglutathione affinity and hydroxyapatite chromatography. This enzyme is not found in all livers; it has been found in only one of twenty specimens examined. When present it accounts for approximately 25% of the total cytosolic GST activity. Glutathione S-transferase ϕ was purified from the transferase fraction of cytosol which bound with high affinity to DEAE-cellulose at pH 8.1 (Pool 3). It comprises two subunits of identical size, (Mr 26 700, Fig. 1) similar to the single Yb-containing enzyme form (GST μ) described by Warholm et al. (1983). Antisera raised to GST μ cross-reacts with GST ϕ using immuno-blotting analysis. The isoelectric point of GST ϕ is pI 4.6 (Fig. 2) and is distinct from that of GST μ (pI 6.1). GST ϕ has a 10-fold lower activity with ethacrynic acid as substrate than GST μ and is approximately 50% less active with substrates 1-chloro-2,4-dinitrobenzene and trans-4-phenyl-3-buten-2-one than GST μ.

REFERENCE

Warholm, M., Guthenberg, C. & Mannervik, B., 1983, Biochemistry, 22, 3610-3617.

Figure 1. SDS/PAGE of GST. Lanes 1 and 7, rat Ya, Yb, Yc subunits; lanes 2-5, human "Basic" (Ya-type) GST; lane 6, GSTϕ (Yb-type).

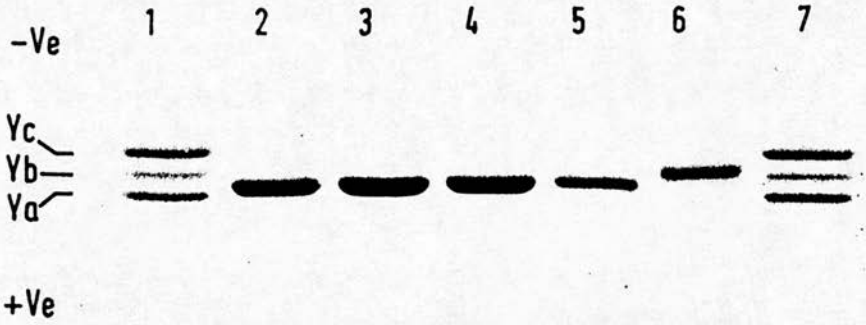
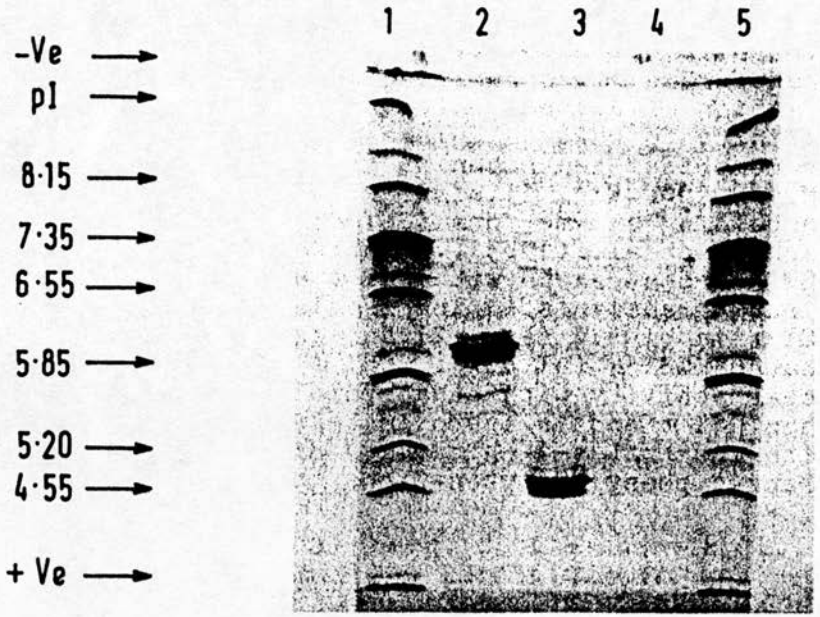


Figure 2. Isoelectric focusing of human GST. Lanes 1 and 5 protein pI markers; lane 2, GSTμ ; lane 3 GSTϕ .



Biochem. J. (1987) **244**, 55–61 (Printed in Great Britain)

Characterization of the basic glutathione *S*-transferase B_1 and B_2 subunits from human liver

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The basic glutathione *S*-transferases in human liver are composed of at least two immunochemically distinct polypeptides, designated B_1 and B_2. These subunits exist as homodimers, but can hybridize to form the B_1B_2 heterodimer [Stockman, Beckett & Hayes (1985) Biochem. J. **227**, 457–465]. Although these basic glutathione *S*-transferases possess similar catalytic properties, the B_2 subunit exhibits significantly greater selenium-independent glutathione peroxidase activity than subunit B_1. The use of the ligands haematin, tributyltin acetate and Bromosulphophthalein as inhibitors of 1-chloro-2,4-dinitrobenzene–GSH-conjugating activity clearly discriminate between the B_1 and B_2 subunits and should help facilitate their identification. Peptide mapping experiments showed that B_1 and B_2 are structurally distinct, but related, subunits; subunit B_1 yielded 43 tryptic peptides, seven of which were unique, whereas subunit B_2 yielded 40 tryptic peptides, four of which were unique.

INTRODUCTION

The cytosolic glutathione *S*-transferases (GSTs) are a complex supergene family of dimeric enzymes (Tu & Reddy, 1985; Hayes, 1986; Rothkopf *et al.*, 1986; Li *et al.*, 1986; Ding *et al.*, 1986) that provide the body with several protective mechanisms against toxic xenobiotics (Smith *et al.*, 1977; Chasseaud, 1979).

Like the cytosolic enzymes in the rat, the human GSTs have been divided into three classes, which have been designated basic, neutral and acidic (Awasthi *et al.*, 1980; Warholm *et al.*, 1983; Mannervik, 1985). The basic, neutral and acidic GST enzymes contain polypeptides that not only are immunochemically related to the rat Ya-, Yb- and Yf-type subunits but possess similar mobilities during SDS/polyacrylamide-gel electrophoresis (Hayes & Mantle, 1986*b*,*c*; Tu *et al.*, 1986).

Kamisaka *et al.* (1975) originally described a purification scheme for five basic GST forms (α, β, γ, δ and ϵ) from human liver, which differed in isoelectric point (pI 7.8–8.8) but were indistinguishable by other criteria. These workers proposed that GST α–ϵ were coded by a single gene and that the multiple forms were generated by a post-synthetic modification (Jakoby & Habig, 1980). We have since demonstrated that the basic human hepatic GST enzymes contain two immunochemically distinct subunits (Hayes *et al.*, 1983; Stockman *et al.*, 1985), designated B_1 and B_2; these have been shown to hybridize *in vitro*. The B_1B_1, B_1B_2 and B_2B_2 forms probably correspond to GST ϵ, δ and γ (Stockman *et al.*, 1985). Some uncertainty surrounds the original identity of GST α–ϵ (Stockman *et al.*, 1985), and as a result of the polymorphism associated with these enzymes in man it is possible that further basic GST subunits exist (Hussey *et al.*, 1986).

During the present study we have examined the catalytic and structural properties of GST B_1B_1, B_1B_2 and B_2B_2, to determine the significance of the immunochemical differences reported for the B_1 and B_2 GST subunits. The data presented indicate that the inter-individual differences in the isoenzyme composition of basic GST will be reflected in inter-individual differences in GST activities.

MATERIALS AND METHODS

Chemicals

These were all obtained commercially. Haematin, Cibacron Blue, Bromosulphophthalein, lithocholic acid 3-sulphate and cholic acid were from Sigma Chemical Co., Poole, Dorset, U.K. Cholic acid was purified before use by recrystallization, three times, from ethanol. Triethyltin bromide, tributyltin acetate and triphenyltin chloride were purchased from ICN Biomedicals, K & K Laboratories Division, Plainview, NY, U.S.A. *S*-Hexylglutathione was synthesized by the method of Vince *et al.* (1971). The *S*-hexylglutathione affinity matrix was made by coupling the γ-glutamyl moiety of *S*-hexylglutathione to epoxy-activated Sepharose 6B (Mannervik & Guthenberg, 1981).

Analytical

The electrophoretic methods used have been described previously (Stockman *et al.*, 1985; Hayes & Mantle, 1986*c*).

The amino acid compositions were kindly determined by Dr. R. P. Ambler, Department of Molecular Biology, University of Edinburgh.

Tryptic peptide mapping experiments were carried out on performic acid-oxidized protein by using methods devised by Ambler (1963) and Ambler & Brown (1967).

The antibodies used during the present study, and the immunization schedule by which they were obtained, have been described previously (Hayes *et al.*, 1983; Beckett & Hayes, 1984).

Abbreviation used: GST, glutathione *S*-transferase.

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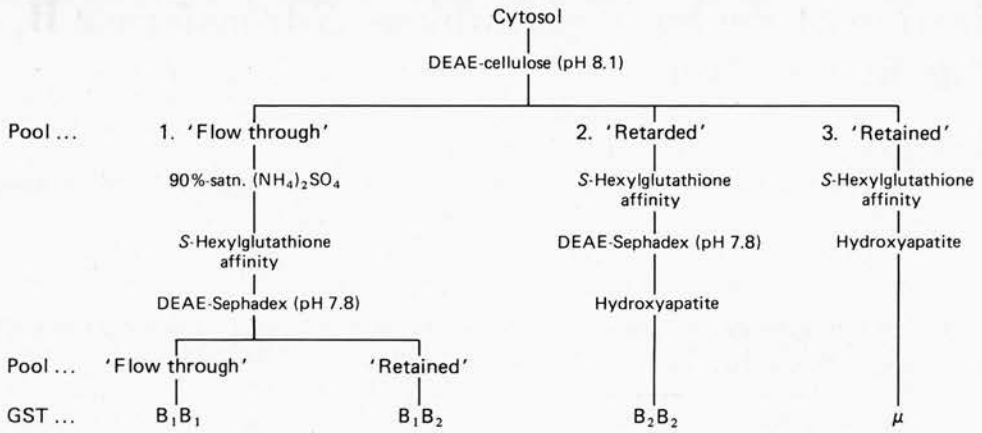

Scheme 1. Purification of human GST enzymes

Purification of hepatic GST

Human livers were obtained within 8 h of death, at autopsy, from individuals (45–69 years old) who had died from disease processes that did not involve the liver.

The basic enzymes, B_1B_1, B_1B_2 and B_2B_2, and the neutral enzyme, GST μ, were isolated from human liver cytosol by using standard chromatographic procedures (Hayes *et al.*, 1983, 1987; Stockman *et al.*, 1985). A summary of the purification strategy used is shown in Scheme 1.

Purification of extrahepatic GST

An acidic class transferase, GST λ, was isolated from human lung by methods described elsewhere (Hayes *et al.*, 1987).

RESULTS

Polymorphism

The majority of the livers studied to date have been found to contain large amounts of GST B_1B_1 and B_1B_2. Relatively few livers were found to express significant amounts of GST B_2B_2; the liver from which this enzyme was isolated provided a yield of about 3 mg of B_2B_2 protein per 100 g of tissue. As already noted by Warholm *et al.* (1983) the neutral enzyme, GST μ, is found in only about 60% of liver specimens.

Electrophoretic properties of human GST

Portions of the basic, neutral and acidic GST enzymes were analysed by SDS/polyacrylamide-gel electrophoresis and isoelectric focusing. The basic subunits, B_1 and B_2, co-migrated during SDS/polyacrylamide-gel electrophoresis as single bands that ran between the Ya (M_r 25500) and Yb (M_r 26300) monomers from rat liver. Both B_1 and B_2 subunits have an apparent M_r of about 26000. The subunits from GST μ migrated between the Yb (M_r 26300) and Yc (M_r 27500) monomers from the rat; the neutral subunits have an M_r of 26700. The subunits from GST λ co-migrated with the rat Yf monomer; the acidic subunit has an M_r of about 24800.

Isoelectric focusing of GST B_1B_1, B_1B_2 and B_2B_2 revealed that these enzymes had pI values of 8.9, 8.75 and 8.4 respectively, whereas GST μ and λ had pI values of 6.1 and 4.8 respectively. These data indicate that the GST enzymes obtained during the present study were homogeneous and closely similar to forms isolated previously in this laboratory from separate human liver specimens.

Substrate specificities of human GST

Table 1 lists the specific activity of each human liver enzyme for various GST substrates; also included for comparison are the values obtained for the acidic transferase from human lung, GST λ. All of the enzymes studied were highly active with 1-chloro-2,4-dinitro-

Table 1. Specific activities of human GST enzymes

N.D. denotes values that were not determined (because of shortage of material).

Substrate	GST ...	Specific activity (μmol/min per mg at 37 °C)				
		B_1B_1	B_1B_2	B_2B_2	μ	λ
1-Chloro-2,4-dinitrobenzene		82	117	80	272	212
1,2-Dichloro-4-nitrobenzene		0.25	0.86	0.79	0	0.14
Ethacrynic acid		0.11	0.16	0.14	0.22	1.22
trans-4-Phenylbut-3-en-2-one		0	0	0	0.45	0.02
1,2-Epoxy-3-(*p*-nitrophenoxy)propane		0	0	0	N.D.	0.53
Cumene hydroperoxide		31	92	104	N.D.	0.11
p-Nitrophenyl acetate		0.66	0.97	0.24	N.D.	0.38

Table 2. Inhibition of human basic GST enzymes

The inhibition of GST activity was studied with 1 mM-1-chloro-2,4-dinitrobenzene and 2 mM-GSH as substrates at pH 6.5 and 37 °C. The I_{50} value is the concentration of inhibitor that decreased GST activity (under these conditions) to 50% (see, e.g., Hayes & Mantle, 1986*a*). Triethyltin bromide is listed first since it is the only compound tested that inhibited the B_2 subunit more than the B_1 subunit.

Inhibitor	GST ...	I_{50} value (μM) B_1B_1	B_1B_2	B_2B_2
Triethyltin bromide		1.55	0.76	0.145
Tributyltin acetate		< 0.001	0.15	0.98
Triphenyltin chloride		0.3	0.7	1.5
Haematin		1.5	3	40
Cibacron Blue		2.5	7.8	24
S-Hexylglutathione		4.6	5.6	6.6
Bromosulphophthalein		10.5	20	125
Lithocholic acid 3-sulphate		6.6	28	36
Cholic acid		1.15×10^3	2.4×10^3	4.1×10^3

benzene, but some enzyme forms exhibited little or no detectable activity with other substrates. Of particular interest was the high glutathione peroxidase activity, with cumene hydroperoxide as substrate, of GST B_1B_1, B_1B_2 and B_2B_2. The B_1 and B_2 subunits appear to possess significantly different glutathione peroxidase II activities: the B_2 subunit exhibits a 3-fold greater activity for cumene hydroperoxide than does the B_1 subunit. The basic enzymes were less active with 1-chloro-2,4-dinitrobenzene but more active with 1,2-dichloro-4-nitrobenzene than were the other enzymes. By contrast, GST μ was characterized by a high specific activity with *trans*-4-phenylbut-3-en-2-one but was inactive with 1,2-dichloro-4-nitrobenzene. None of these enzymes displayed a high specific activity with ethacrynic acid, the property that is characteristic of GST λ.

It should be noted that the hybrid basic GST, B_1B_2, possessed a significantly greater specific activity for substrates such as 1-chloro-2,4-dinitrobenzene and *p*-nitrophenyl acetate than did either of the homodimers, B_1B_1 and B_2B_2.

Inhibition of basic GST activity

Table 2 shows the I_{50} results for the three basic GST enzymes with a variety of inhibitors. The organometal halides were the most potent of all the inhibitors, the greatest inhibition being produced by tributyltin acetate. GST B_1B_1, B_1B_2 and B_2B_2 did not respond equally to the effects of each inhibitor. Small but significant differences (up to 6-fold) in the degree of inhibition of GST B_1B_1 compared with GST B_2B_2 were found with cholic acid, lithocholic acid 3-sulphate, *S*-hexylglutathione and triphenyltin chloride. However, tributyltin acetate was at least 1000 times more effective at inhibiting the activity of GST B_1B_1 than that of GST B_2B_2. Haematin was about 30 times more effective with GST B_1B_1 than with GST B_2B_2, and Cibacron Blue and Bromosulphophthalein also produced similar but less marked differences in the level of inhibition. Triethyltin bromide inhibited GST B_2B_2 about 10 times more than it did GST B_1B_1, and was the only compound tested that was more effective with GST B_2B_2 than GST B_1B_1.

All the I_{50} values obtained for the GST B_1B_2 heterodimer were found to lie between those of the corresponding homodimers, reflecting the catalytic contribution of the two functionally distinct subunits to the hybrid enzyme.

Table 3. Amino acid composition of human hepatic GST enzymes

Samples (0.5 mg of protein) were hydrolysed in 6 M-HCl for 24 h. The results were obtained in μmol of amino acid recovered and from these values were calculated the percentage amino acid composition of each protein. The recoveries of cysteine and tryptophan were not determined and have been excluded from the calculations. The compositions were not corrected for the increased or decreased recoveries of serine, threonine, valine and isoleucine. Data for B_1B_1 GST and B_1B_2 GST were not significantly different from those for B_2B_2 GST and are not shown.

Amino acid	GST ...	Amino acid composition (%) B_2B_2	μ
	pI ...	8.4	6.1
	Subunit M_r ...	26000	26700
Lys		10.8	10.2
His		1.3	2.4
Arg		5.1	4.7
Asx		8.4	11.2
Thr		1.9	3.1
Ser		6.3	4.9
Glx		13.3	6.3
Pro		4.6	5.1
Gly		5.2	5.3
Ala		6.4	4.6
Val		3.9	3.4
Met		3.4	3.6
Ile		6.9	6.9
Leu		13.6	13.2
Tyr		4.4	6.0
Phe		4.4	6.4

Amino acid compositions

All the basic enzymes studied were found to have closely similar amino acid compositions and could not be

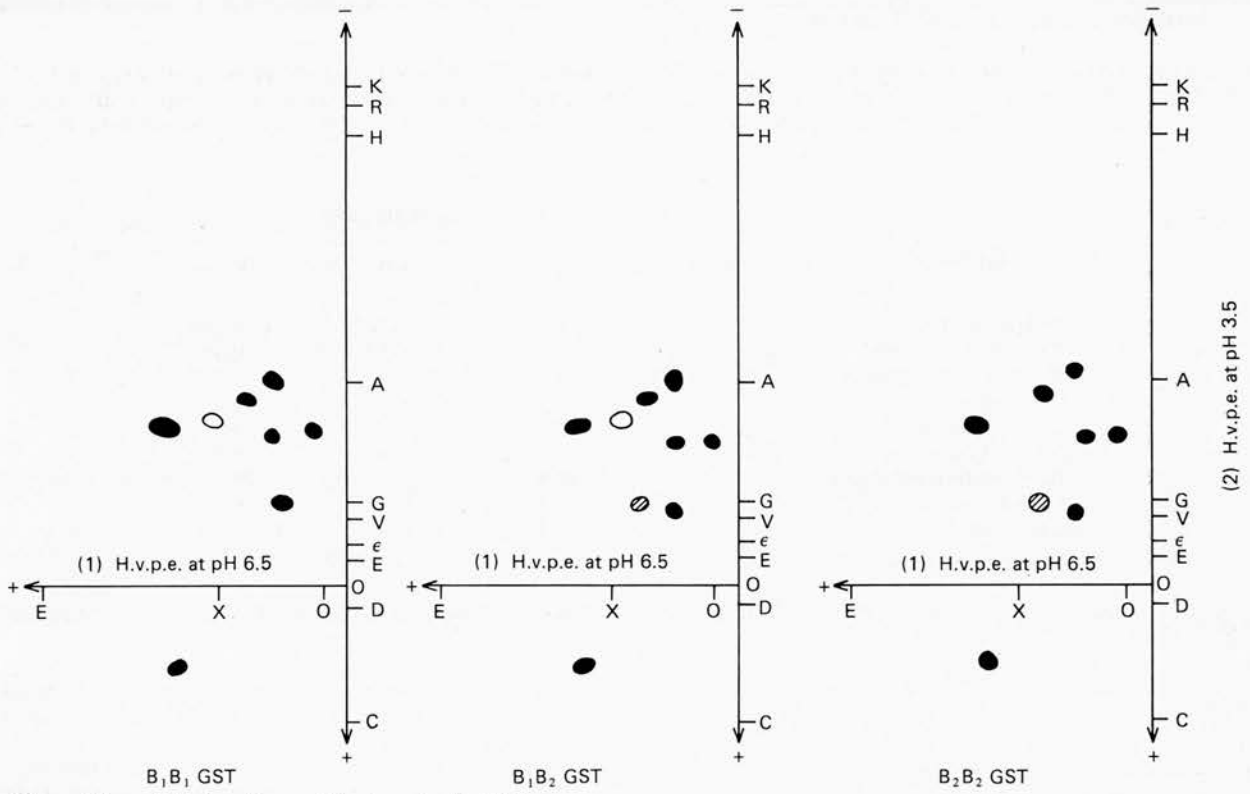

Fig. 1. Map of the acidic tryptic peptides from basic GST enzymes

The tryptic digests of portions (1.5 mg of protein) of B_1B_1, B_1B_2 and B_2B_2 GST enzymes were prepared. Peptides were separated by high-voltage paper electrophoresis (h.v.p.e.) at pH 6.5 in the first dimension. The acidic peptides were cut out of the paper and separated in the second dimension by high-voltage paper electrophoresis at pH 3.5. The stained peptide spots were traced to produce the diagram. A mixture of amino acids (Ambler, 1963) was run in parallel to facilitate comparisons between the maps; the abbreviations employed to indicate the mobilities of these markers have been described elsewhere (Hayes, 1986). Peptides common to all three GST enzymes are represented by blackened spots. The peptide that appeared unique to the B_1 subunit is not shaded. The peptide that appeared unique to the B_2 subunit is shown by the hatched spot.

discriminated on this basis. However, the recoveries of tyrosine, phenylalanine, aspartic acid and glutamic acid obtained from the neutral enzyme, GST μ, were significantly different from the values obtained from the basic GST forms (Table 3).

Peptide mapping of basic GST enzymes

Diagrammatic representations of the acidic, neutral and basic tryptic peptides obtained from oxidized portions of B_1B_1, B_1B_2 and B_2B_2 proteins are shown in Figs. 1–3.

Examination of the maps of the acid peptides (Fig. 1) revealed that seven were common to GSH B_1B_1, B_1B_2 and B_2B_2. One acidic peptide (no shading) was found only in the B_1-containing GST enzymes, B_1B_1 and B_1B_2. A further acidic peptide (hatched shading) was only recovered from the B_2-containing GST enzymes, B_2B_2 and B_1B_2.

The neutral peptide maps (Fig. 2) demonstrated the existence of ten peptides shared by GST B_1B_1, B_1B_2 and B_2B_2. Four additional tryptic peptides were observed in GST B_1B_1 and B_1B_2 (no shading), but not in GST B_2B_2. Conversely, three peptides were found in GST B_1B_2 and B_2B_2 (hatched shading), but not in GST B_1B_1.

The basic peptide maps (Fig. 3) revealed 19 tryptic peptides common to GST B_1B_1, B_1B_2 and B_2B_2. Two additional peptides appear to be specific for the B_1 subunit (no shading); no basic peptides were recovered that were specific for the B_2 subunit.

The tryptic peptides that appeared to be specific for the B_1 and B_2 subunits, the 'difference peptides', occurred in a manner consistent with the hypothesis that GST B_1B_2 is a heterodimer comprising a subunit common to GST B_1B_1 and a subunit common to GST B_2B_2. No peptides were found to be unique to any one of the basic GST enzymes.

Immunoblotting

The polyclonal antisera previously employed in radioimmunoassays to discriminate between B_1 and B_2 polypeptides (Beckett & Hayes, 1984; Stockman *et al.*, 1985) were used to probe these subunits following transfer to nitrocellulose. Neither anti-(B_1 subunit) nor anti-(B_2 subunit) serum could distinguish between the two basic subunits in Western-blotting experiments, despite their 300–500-fold difference in cross-reactivity in the radioimmunoassays. This method-dependent difference in immunoreactivity reflects the fact that the radioimmunoassay technique is used to challenge GST their native, active, state, whereas the Western-blot technique probes denatured GST subunits in random-coil configuration. These data are consistent with the close sequence homology between subunits B_1 and B_2 that is evident from the peptide 'mapping' experiments.

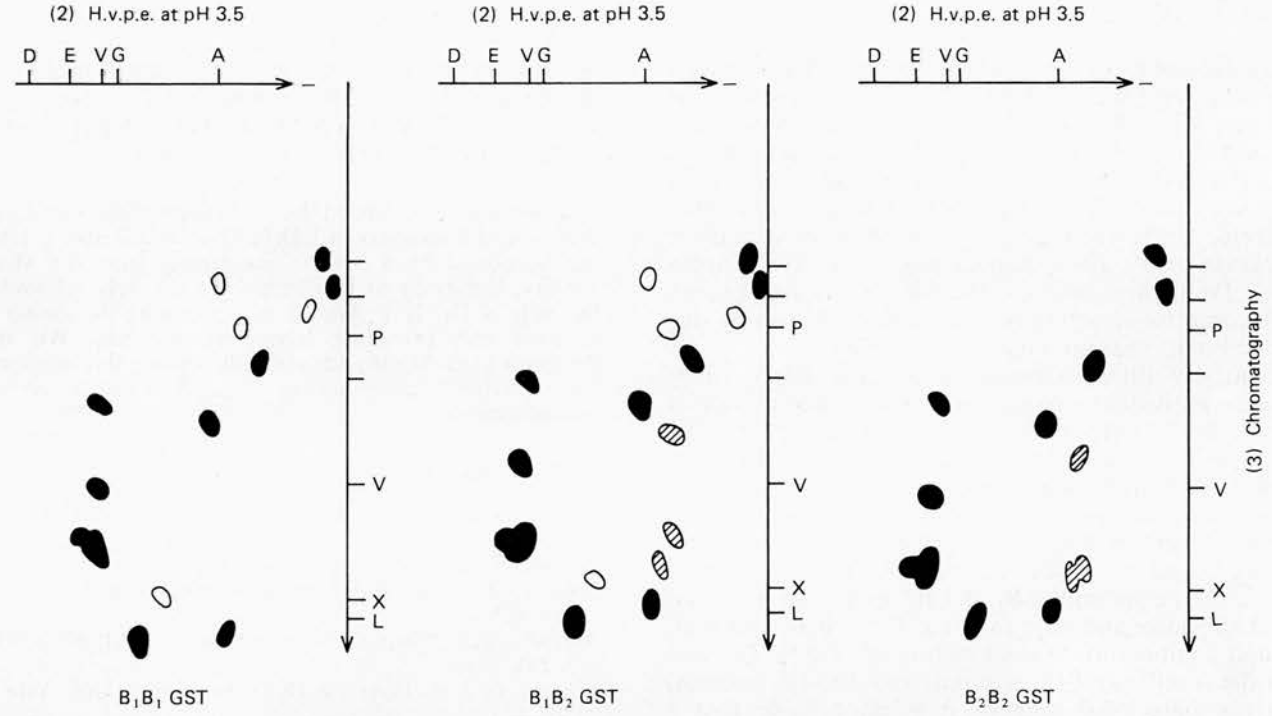

Fig. 2. Map of the neutral tryptic peptides from basic GST enzymes

The neutral tryptic peptides from portions (1.5 mg of protein) of B_1B_1, B_1B_2 and B_2B_2 GST enzymes were isolated by high-voltage paper electrophoresis (h.v.p.e.) at pH 6.5. These were resolved in the first dimension by high-voltage paper electrophoresis at pH 3.5. Separation in the second dimension was performed by descending chromatography using butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) for 16 h. The stained peptide spots were traced to produce the diagram. Peptides common to all 'basic' GST enzymes are represented by blackened spots. The four neutral peptides that appeared unique to the B_1 subunit are not shaded, and the three neutral peptides unique to the B_2 subunit are shown by the hatched spots.

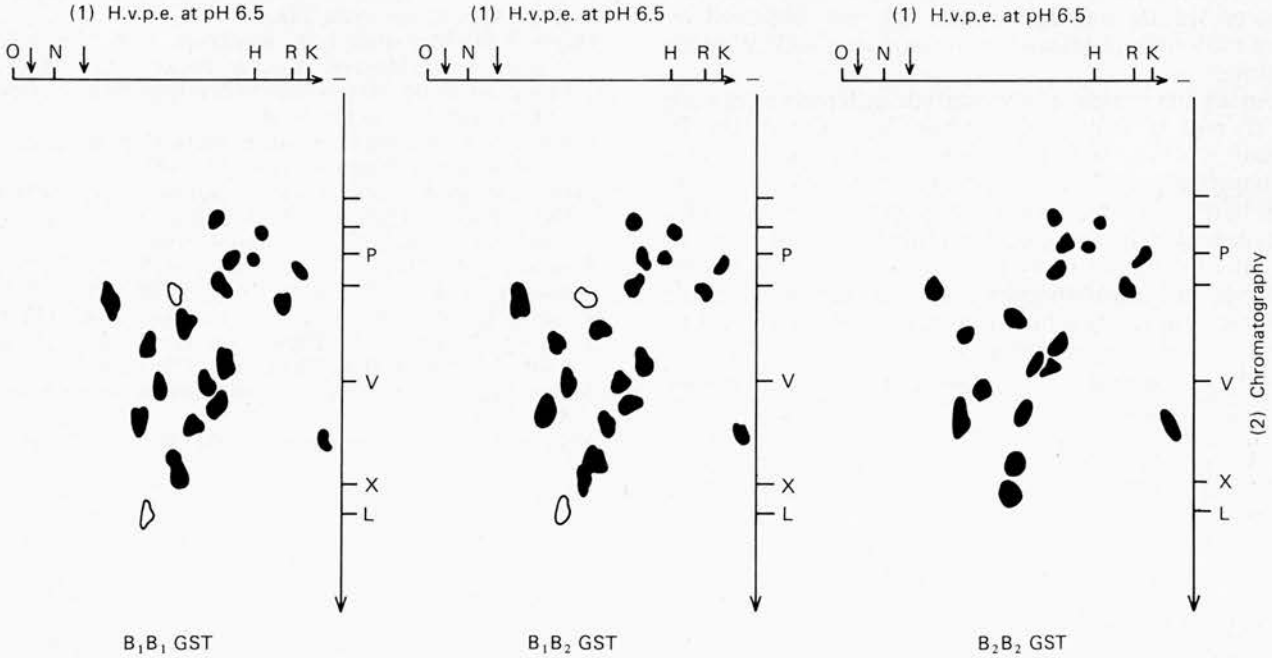

Fig. 3. Map of the basic tryptic peptides from basic GST enzymes

The map of the basic tryptic peptides was constructed by high-voltage paper electrophoresis (h.v.p.e.) at pH 6.5 in the first dimension, and descending chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) in the second dimension. The ninhydrin-positive peptides were traced to produce the diagram. The blacked spots represent common peptides, and the spots that are not shaded represent peptides unique to the B_1 subunit.

DISCUSSION

The cytosolic GST enzymes in man have been divided into three groups, which are thought to represent the products of different gene families; these have been referred to, according to their pI values, as the basic, the neutral and the acidic GST. These genetic relationships have been concluded from data (Warholm *et al.*, 1983; Koskelo, 1983; Dao *et al.*, 1984; Ålin *et al.*, 1985; Stockman *et al.*, 1985; Vander Jagt *et al.*, 1985; Soma *et al.*, 1986) that indicated the three classes of human GST comprise structurally and immunochemically distinct subunits that are functionally separate.

Relatively little is known about the relationships between individual enzymes within the three classes of human GST, but, to date, the basic group of GST enzymes has been most extensively studied. Previous work from this laboratory described two antisera that, in radioimmunoassays, could discriminate between members of this group of GST (Beckett & Hayes, 1984). This finding led us to suggest the presence of two separate basic GST polypeptides, B_1 and B_2, in human liver that could hybridize and were found as B_1B_1, B_1B_2 and B_2B_2 subunit combinations (Stockman *et al.*, 1985). The fact that the specific anti-(B_1 subunit) and anti-(B_2 subunit) sera are polyclonal suggests it is improbable that a post-synthetic modification could account for the differences in the B_1 and B_2 subunits.

The peptide 'mapping' experiments described in Figs. 1–3 not only support the hypothesis that a hybrid basic GST exists in man, but also show that the B_1 and B_2 subunits are structurally distinct. These data suggest that the B_1 and B_2 subunits are genetically separate but share a significant sequence homology (possibly $> 90\%$ identity). With this degree of homology it is likely that the 'difference peptides' are exposed on the surface of the protein, since the immunochemical differences that exist between the B_1 and B_2 subunits are only observed by using radioimmunoassay but are not seen with Western blotting.

During the present study catalytic differences between the B_1 and B_2 subunits have been established; the B_2 subunit has been found to exhibit a significantly greater selenium-independent glutathione peroxidase activity than that of the B_1 subunit. Large differences have also been revealed in the susceptibilities of the B_1 and B_2 subunits to inhibition by tributyltin acetate, triethyltin bromide, Bromosulphophthalein and haematin. These differences in the functional properties of the B_1 and B_2 subunits may suggest that at least one of the 'difference peptides' described above reside near the hydrophobic binding domain.

Inhibitors of GST activity have been used to discriminate between related subunits in the rat (Hayes & Mantle, 1986*a*; Tahir & Mannervik, 1986). The inhibitors used in the present study provide a simple means of distinguishing between the two human GST subunits B_1 and B_2. Of all the compounds tested the majority were found to inhibit subunit B_1 more than subunit B_2; tributyltin and haematin showed the largest difference in inhibition of subunit B_1 relative to subunit B_2. By contrast, triethyltin bromide showed a 10-fold greater inhibition of subunit B_2 than of subunit B_1.

Polymorphism of the neutral Yb-containing GST in man has been recognized (Warholm *et al.*, 1980; Board, 1981; Strange *et al.*, 1984; Hussey *et al.*, 1986). Marked variations also exist in the content of basic GST in liver specimens. The differences in the catalytic and ligand-binding activities of the B_1 and B_2 subunits indicate that the basic GST may be responsible for some of the variations within the population in drug metabolism and in susceptibility to cytotoxic compounds.

This work was funded by a Medical Research Council Project Grant awarded to J.D.H. (G8126 392 SB). L.I.McL. was supported by a Bonnar Scholarship from the Medical Faculty, University of Edinburgh. We sincerely acknowledge the help of Dr. R. P. Ambler in performing the amino acid analyses and providing laboratory facilities. We thank Professor L. G. Whitby for critically reading this manuscript. The secretarial assistance of Mrs. E. Ward is gratefully acknowledged.

REFERENCES

Ålin, P., Mannervik, B. & Jörnvall, H. (1985) FEBS Lett. **182**, 319–322
Ambler, R. P. (1963) Biochem. J. **89**, 349–378
Ambler, R. P. & Brown, L. H. (1967) Biochem. J. **104**, 784–825
Awasthi, Y. C., Dao, D. D. & Saneto, R. P. (1980) Biochem. J. **191**, 1–10
Beckett, G. J. & Hayes, J. D. (1984) Clin. Chim. Acta **141**, 267–273
Board, P. G. (1981) Am. J. Hum. Genet. **33**, 36–43
Chasseaud, L. F. (1979) Adv. Cancer Res. **29**, 175–274
Dao, D. D., Partridge, C. A., Kurosky, A. & Awasthi, Y. C. (1984) Biochem. J. **221**, 33–41
Ding, G. J.-F., Ding, V. D.-H., Rodkey, J. A., Bennett, C. D., Lu, A. Y. H. & Pickett, C. B. (1986) J. Biol. Chem. **261**, 7952–7957
Hayes, J. D. (1986) Biochem. J. **233**, 789–798
Hayes, J. D. & Mantle, T. J. (1986*a*) Biochem. J. **233**, 407–415
Hayes, J. D. & Mantle, T. J. (1986*b*) Biochem. J. **233**, 779–788
Hayes, J. D. & Mantle, T. J. (1986*c*) Biochem. J. **237**, 731–740
Hayes, J. D., Gilligan, D., Chapman, B. J. & Beckett, G. J. (1983) Clin. Chim. Acta **134**, 107–121
Hayes, J. D., McLellan, L. I., Stockman, P. K., Chalmers, J., Howie, A. F., Hussey, A. J. & Beckett, G. J. (1987) in European Drug Metabolism Workshop 10th, Taylor and Francis, London, in the press
Hussey, A. J., Stockman, P. K., Beckett, G. J. & Hayes, J. D. (1986) Biochim. Biophys. Acta **874**, 1–12
Jakoby, W. B. & Habig, W. H. (1980) in Enzymatic Basis of Detoxification (Jakoby, W. B., ed.), vol. 2, pp. 63–94, Academic Press, London and New York
Kamisaka, K., Habig, W. H., Ketley, J. N., Arias, I. M. & Jakoby, W. B. (1975) Eur. J. Biochem. **60**, 153–161
Koskelo, K. (1983) Scand. J. Clin. Lab. Invest. **43**, 133–139
Li, N.-Q., Reddanna, P., Thyagaraju, K., Reddy, C. C. & Tu, C.-P. D. (1986) J. Biol. Chem. **261**, 7596–7599
Mannervik, B. (1985) Adv. Enzymol. Relat. Areas Mol. Biol. **57**, 357–417
Mannervik, B. & Guthenberg, C. (1981) Methods Enzymol. **77**, 231–235
Rothkopf, G. S., Telakowski-Hopkins, C. A., Stotish, R. L. & Pickett, C. B. (1986) Biochemistry **25**, 993–1002
Smith, G. J., Ohl, V. S. & Litwack, G. (1977) Cancer Res. **37**, 8–14
Soma, Y., Satoh, K. & Sato, K. (1986) Biochim. Biophys. Acta **869**, 248–258
Stockman, P. K., Beckett, G. J. & Hayes, J. D. (1985) Biochem. J. **227**, 457–465
Strange, R. C., Faulder, C. G., Davis, B. A., Hume, R., Brown, J. A. H., Cotton, W. & Hopkinson, D. A. (1984) Ann. Hum. Genet. **48**, 11–20
Tahir, M. K. & Mannervik, B. (1986) J. Biol. Chem. **261**, 1048–1051

Tu, C.-P. D. & Reddy, C. C. (1985) J. Biol. Chem. **260**, 9961–9964
Tu, C.-P. D., Matsushima, A., Li, N.-Q., Rhoads, D. M., Srikumar, K., Reddy, A. P. & Reddy, C. C. (1986) J. Biol. Chem. **261**, 9540–9545
Vander Jagt, D. L., Hunsaker, L. A., Garcia, K. B. & Royer, R. E. (1985) J. Biol. Chem. **260**, 11603–11610
Vince, R., Daluge, S. & Wadd, W. B. (1971) J. Med. Chem. **14**, 402–404
Warholm, M., Guthenberg, C., Mannervik, B., von Bahr, C. & Glaumann, H. (1980) Acta Chem. Scand. B **34**, 607–610
Warholm, M., Guthenberg, C. & Mannervik, B. (1983) Biochemistry **22**, 3610–3617

Received 16 October 1986/14 November 1986; accepted 23 January 1987

Mouse Liver Tumors
Arch. Toxicol., Suppl. 10, 136–146 (1987)

Glutathione S-Transferase Subunits in the Mouse and Their Catalytic Activities Towards Reactive Electrophiles

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Abstract. The glutathione S-transferases (GST) are a major, multi-gene, group of detoxication proteins. A rapid, two-step purification, that employs S-hexylglutathione affinity chromatography and hydroxyapatite HPLC, is described for the GST isoenzymes in mouse liver. The major hepatic forms comprise Yf(Mr 24 500)-, Ya(Mr 26 000)- and Yb(Mr 27 000)-type subunits. The isoeletric points of the Yf, Ya and Yb dimers are 8.6, 9.2 and 7.8–8.2 respectively. Immunochemical experiments showed the purified mouse subunits cross-reacted with antisera raised against rat GST subunits that possessed equivalent molecular masses; the subunit sizes of the distinct subunit types are conserved between these two species. The catalytic activities of the purified mouse GST subunits are described. In common with other species the mouse GST are subject to tissue-specific expression. However, only mouse liver and not rat, guinea pig, hamster or human livers express the Yf polypeptide. The significance of this difference is unclear but, in the rat, the Yf subunit can serve as a marker for pre-neoplastic hepatocellular carcinogenesis.

Key words: Glutathione S-transferases – Isoenzymes – Hydroxyapatite HPLC – Detoxification – Tumour marker

Introduction

The glutathione S-transferases (GST) represent a complex group of isoenzymes that catalyse the nucleophilic attack by glutathione (GSH) on hydrophobic xenobiotics which possess an electrophilic centre (Jakoby 1978). The GSH/electrophile adducts formed by the actions of GST are, in general, more soluble and less toxic than the electrophilic substrates (Boyland and Chasseaud 1969); these glutathione conjugates may be metabolised further to cysteinyl-glycine conjugates, cysteine conjugates and N-acetylcysteine conjugates (mercapturic acids) before elimination from the body. Collectively, the GST provide protection against a wide range of potentially toxic drugs and foreign compounds (Chasseaud 1976;

Mannervik 1985) and represent an important defence mechanism against chemical carcinogenesis (Smith et al. 1977; Sparnins et al. 1982).

GST have been widely studied in the rat but much less is known of the forms in other species. The cytosolic GST are dimeric and the rat enzymes are composed of Yf(Mr 24 500)-, Yk(Mr 25 000)-, Ya(Mr 25 500)-, Yn(Mr 26 500)-, Yb(Mr 27 000)- and Yc(Mr 28 500)-type subunits (Hayes and Chalmers 1983; Hayes 1986). The Ya- and Yc-type subunits and the Yn- and Yb-type subunits can hybridize forming heterodimers (Hayes et al. 1981; Hayes 1984). The GST that comprise Yf-, Ya-, Yn-, Yb- and Yc-type subunits can be isolated by S-hexylglutathione affinity chromatography whilst the Yk subunit binds this matrix weakly. The latter can be isolated by glutathione affinity chromatography (Hayes 1986).

The GST subunits function autonomously (Jakobson et al. 1977) and, as each monomer is active towards a distinct spectrum of electrophiles, individual subunits appear to serve different detoxification roles (Glatt et al. 1983; Coles et al. 1985; Jernstrom et al. 1985).

In the rat the GST subunits are subject to tissue-specific expression (Hayes and Mantle 1986). For example, the Yf polypeptide is not normally found in rat liver but is found in hepatic pre-neoplastic foci (Faber 1984; Satoh et al. 1985; Meyer et al. 1985; Jensson et al. 1985). Most extra-hepatic tissues, with the exception of testis, contain significant amounts of Yf (Hayes and Mantle 1986).

Few data have been published about GST in the mouse (for purification schemes, see Lee et al. 1981; Lee 1982; Pearson et al. 1983; Agius and Gidari 1985), and little is known about the subunits expressed in this animal.

In the present communication the electrophoretic and immunochemical properties of the GST subunits in the mouse are described. A rapid purification scheme has been devised for the hepatic GST isoenzymes, and their catalytic activities as well as subunit compositions have been investigated. The expression of GST subunits in extra-hepatic tissues is also described.

Materials and Methods

Chemicals and Tissues

The chemicals used were of analytical grade and readily available commercially. The construction of the S-hexylglutathione and the glutathione affinity matrices, using epoxy-activated Sepharose 6B, have been described by Mannervik and Guthenberg (1981) and Simons and Vander Jagt (1977) respectively.

The tissues used for enzyme purification were from random bred male LACA mice fed *ad libitum* and housed in the Department of Biochemistry, Trinity College, Dublin. The livers were frozen at $-85\,^{\circ}C$, for 3 months, prior to use. Extra-hepatic tissues were processes immediately.

Enzyme Purification

Mouse GST were isolated from the $100\,000 \times g$ supernatant fraction of liver, kidney, testis and lung. Extracts, 25% (w/v), of these tissues were prepared in 20 m*M*-Tris/HCl buffer pH 7.8 that contained 200 m*M* NaCl. Following centrif-

ugation the cytosols were extensively dialysed against the same buffer before being subjected to chromatography on columns (1.6 cm × 15 cm) of S-hexylglutathione-Sepharose 6B. This matrix retained more than 85% of the GST in the mouse, and the material specifically purified by S-hexylglutathione affinity chromatography could therefore be analysed directly by "Western blotting" (Towbin et al. 1979) to identify individual GST subunits. The same strategy was employed to purify the GST from Wistar rat, guinea pig, golden hamster and postmortem human liver; these enzyme pools were prepared to enable mouse GST subunits to be compared with those in other species.

To allow the possibility that additional mouse GST subunits exist that cannot be purified by S-hexylglutathione affinity chromatography, the 1-chloro-2,4-dinitrobenzene-GSH-conjugating activity in mouse liver, that was not retained by S-hexylglutathione-Sepharose 6B, was applied to a glutathione-Sepharose 6B affinity column (for details, see Hayes 1986).

The individual GST isoenzymes in mouse liver, purified by S-hexylglutathione affinity chromatography, were resolved by hydroxyapatite using Bio-Gel HPHT, a HPLC-grade column (from Bio-Rad Laboratories, Watford, Herts, UK). The S-hexylglutathione-Sepharose 6B-purified material was dialysed for 16 h at 4 °C against 2 changes, each of 2 litres, of 10 m*M*-sodium phosphate buffer pH 6.7 containing 2-mercaptoethanol (2 m*M*). The dialysed material (15 ml, 95 mg of protein) was centrifuged (60 min at 150000 × g and 4 °C) before chromatography. The Bio-Gel HPHT column was eluted at 0.5 ml/min and developed using a 10–350 m*M* gradient of sodium phosphate buffer, pH 6.7; the eluent contained 2-mercaptoethanol (2 m*M*) and $CaCl_2$ (0.4 m*M*) throughout. The HPLC system was from Waters Associates (Instruments), Northwich, Cheshire, UK, and comprised two model 510 pumps, a model 680 automated gradient controller, a model 481 Lambda-Max absorbance detector and a model U6K universal injector. Peaks were detected by U.V. absorption at 280 nm.

Analytical

SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) was carried out by the method of Laemmli (1970). The catalytic properties of the purified proteins were examined at 37 °C using enzyme assays for a range of electrophilic substrates (Habig and Jakoby 1981). The immunochemical properties of individual GST isoenzymes was determined by "dot-blotting" 1 μg portions of purified protein on to nitrocellulose. The immobilised proteins were challenged with antiserum against either Yf-, Ya-, Yb- or Yc-type GST subunits purified from the rat (Hayes 1984, 1986). Cross-reacting mouse GST were identified by using the peroxidase-labelled second antibody method (Bio-Rad Laboratories, Watford, Herts, UK) that is also employed for the "Western blot" analysis (Hayes and Mantle 1986).

Results

The hepatic glutathione S-transferases were purified separately from rat and mouse, as well as guinea pig, hamster and man, by S-hexylglutathione affinity chromatography. SDS/PAGE showed that significant differences exist in the

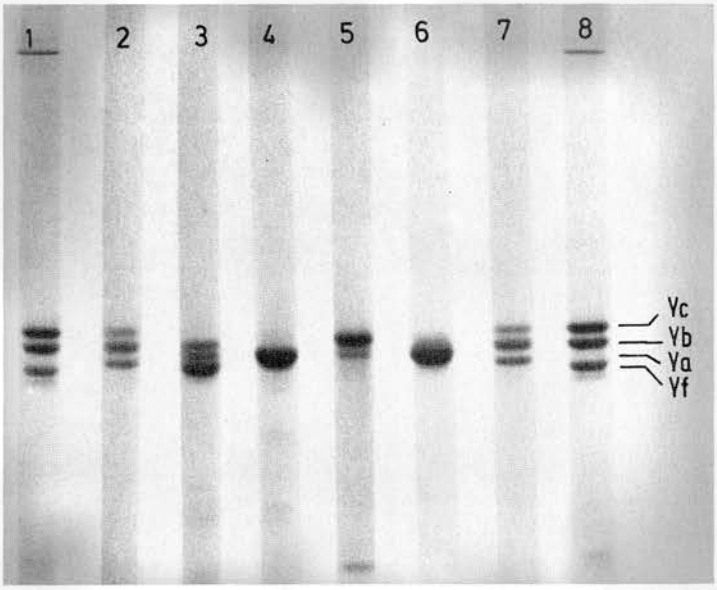

Fig. 1. Comparison of hepatic GST subunits in different species. SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) using a 12.5% (w/v) resolving gel. The transferases were purified by S-hexylglutathione affinity chromatography. The rat Yf (Mr 24 500), Ya (Mr 25 500), Yb (Mr 27 000) and Yc (Mr 28 500) subunits are indicated. The gel was loaded with 5 μg GST in each track as follows: 1 and 8, rat lung GST; 2 and 7, rat liver GST, 3, mouse liver GST; 4, guinea pig liver GST; 5, hamster liver GST; 6, human liver GST

GST subunits expressed in the livers of these species (Fig. 1). In particular, the mouse was the only species examined that contained significant levels of the Yf polypeptide in normal liver; in the rat this subunit is only found in pre-neoplastic nodules (Faber 1984; Meyer et al. 1985). Mouse liver also contained Ya- and Yb-type subunits but little, if any, Yc-type subunits. These results were confirmed immunochemically by "Western blotting" experiments.

The sequential use of S-hexylglutathione and glutathione affinity chromatography demonstrated that a portion of the GST activity that failed to bind the S-hexylglutathione-Sepharose 6B column can be isolated by glutathione-Sepharose 6B chromatography. In the mouse 4.4% of the hepatic cytosolic protein specifically bound S-hexylglutathione-Sepharose 6B whilst 0.2% of the protein that eluted in the "flow-through" fractions from this matrix was isolated by glutathione-Sepharose 6B chromatography. By contrast, only 2.5% of the cytosolic protein in rat liver bound the S-hexylglutathione affinity matrix and the subsequent use of glutathione-Sepharose resulted in a further 0.2% of the cytosolic protein binding the second affinity matrix. It therefore appears that the GST represent a larger portion of the hepatic cytosol in the mouse. Figure 2 shows that the two GST pools from rat liver comprise polypeptides of broadly similar Mr. However, it is apparent that the subunit eluted from S-hexyl-glutathione-Sepharose 6B (first affinity step, GST Pool 1) with the fastest anodal mobility possesses a significantly greater Mr than the subunit from glutathione-Sepharose 6B with the fas-

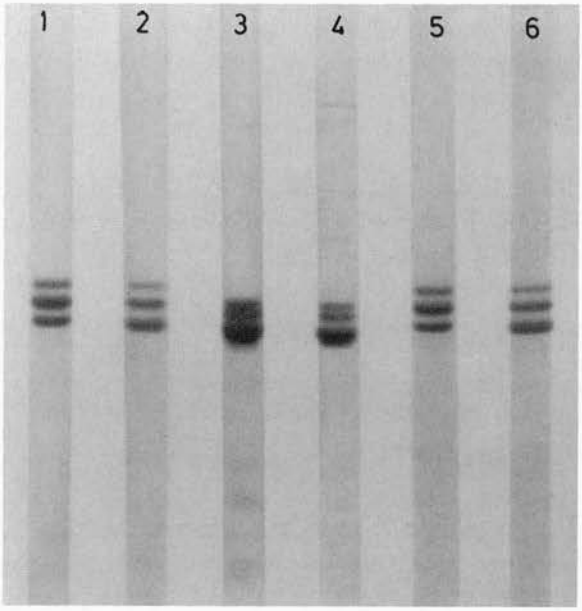

Fig. 2. Electrophoretic analysis of GST purified using S-hexylglutathione- and glutathione-affinity chromatography. Cytosol from rat and mouse liver were separately subjected sequentially to S-hexylglutathione-affinity chromatography and glutathione-affinity chromatography. The GST that bound S-hexylglutathione-Sepharose 6B (GST Pool 1) and the GST that failed to bind S-hexylglutathione-Sepharose 6B but bound glutathione-Sepharose 6B (GST Pool 2) were analysed by SDS/PAGE. The gel was loaded as follows: tracks 1 and 5, rat liver GST Pool 1 (Ya, Yb, Yc); tracks 2 and 6, rat liver GST Pool 2 (Yk, Yb, Yc); track 3, mouse liver GST Pool 1; track 4, mouse liver GST Pool 2

test mobility (GST Pool 2). Similar differences in the electrophoretic band patterns of the two mouse GST Pools 1 and 2 were also observed.

The GST isoenzymes in mouse liver were purified using a combination of S-hexylglutathione affinity chromatography and HPLC-grade hydroxyapatite on Bio-Gel HPHT. Typically, about 2.3 g of cytosol protein (specific activity with 1-chloro-2,4-dinitrobenzene, 3.3 μmol product/min/mg protein) was applied to S-hexylglutathione-Sepharose 6B and resulted in the purification of approx. 95 mg of GST protein (specific activity with 1-chloro-2,4-dinitrobenzene 153 μmol product/min/mg protein). Portions (1 ml, 5–15 mg of protein) of this affinity-purified pool were applied to the Bio-Gel HPHT column. Figure 3 shows the profile observed from the final HPLC, step; 4 main peaks and 1 minor peak were resolved by hydroxyapatite. The Bio-Gel HPHT column was eluted at 0.5 ml/min and peaks 1–5 appeared at 31.5, 36.0, 38.7, 44.0 and 55.0 min respectively. A poorly-resolved shoulder that eluted (at 52.0 min) between peaks 4 and 5 was not studied due to paucity of material. The GST obtained by this two-step strategy were highly purified and the recovery (>85%) was good.

The GST in these peaks were designated P1, P2, P3, P4 and P5 by their order of elution. The specific activities of these enzymes for a range of electrophiles is shown in Table 1. GST P1, P2 and P3 exhibited similar catalytic properties whereas P4 and P5 each possessed distinct activities.

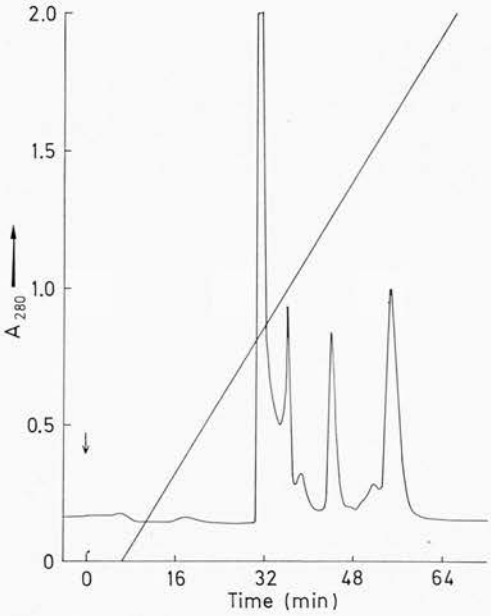

Fig. 3. Use of hydroxyapatite HPLC, to resolve mouse liver GST. A portion (1 ml, 6 mg protein) of GST purified, by S-hexylglutathione affinity chromatography, from mouse liver cytosol was dialysed against 10 m*M*-sodium phosphate buffer pH 6.7 and subjected to chromatography on Bio-Gel HPHT. The hydroxyapatite column was also equilibrated with 10 m*M*-sodium phosphate buffer pH 6.7 and the proteins were eluted (0.5 ml/min) with a linear 10 m*M*–350 m*M*-sodium phosphate gradient that contained 2 m*M*-2-mercaptoethanol and 0.4 m*M*-$CaCl_2$ throughout. The A_{280} of the column eluate was monitored. The minor peak that eluted at 52.0 min was not studied. The GST eluted at 31.5, 36.0, 38.7, 44.0 and 55.0 min are designated P1–P5 respectively

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) (Fig. 4) showed that the GST peaks 1 and 2 contained subunits with Mr 24 500, peak 3 contained polypeptides with Mr 24 000 and 24 500, peak 4 contained subunits with Mr 26 000 and peak 5 comprised monomers of Mr 27 000. Peaks 1, 2 and 3 cross-reacted with anti-Yf-IgG, peak 4 cross-reacted with anti-Ya-IgG, and peak 5 cross-reacted with anti-Yb_1-IgG (Table 2). The protein in peaks 1 and 2 possessed identical isoelectric points (pI 8.6) whereas the protein in peak 4 had a pI $\geqq$ 9.2 and that in peak 5 had a pI 7.8–8.2.

The tissue-specific expression of GST subunits was examined by affinity chromatography and SDS/PAGE. Electrophoresis (Fig. 5) revealed that whilst the liver contains high levels of the Yf subunit (Mr 24 500) this polypeptide is absent, or only present in low levels, in mouse kidney, testis and lung. The extrahepatic tissues contain predominantly Yb-type subunits. Mouse lung contains an additional polypeptide, not seen in the liver, that co-migrates with the rat Yc subunit (Mr 28 500), and mouse kidney contains the low Mr subunit (Mr 24 000) found in the hepatic GST P3 preparation.

Table 1. Catalytic properties of the hepatic glutathione S-transferases. All analyses were performed at 37°C. GST P1–P5 were from mouse liver. GST L (YaYa), A (Yb_1Yb_1) and AA (YcYc) were from rat liver and are included to facilitate comparisons between the GST of the two species.

Substrate	Specific activity (μmol/min/mg protein) Glutathione S-transferase							
	Mouse					Rat		
	P1	P2	P3	P4	P5	YaYa	Yb_1Yb_1	YcYc
1-chloro-2,4-dinitrobenzene	130	115	100	35	181	96	186	51
ethacrynic acid	3.2	3.4	3.4	0.6	0.3	0.4	0.0	3.5
p-nitrobenzyl chloride	1.1	1.4	2.0	0.6	12.0	1.5	14.0	0.3
1,2-dichloro-4-nitrobenzene	0.2	0.2	0.3	0.2	6.5	0.1	10.7	0.1
Δ^5-androstene-3,17-dione	0.2	0.2	0.1	0.0	0.0	2.4	0.0	0.0
cumene hydroperoxide	0.04	0.0	0.05	31.7	0.3	3.6	0.1	11.7

Table 2. Physical and immunochemical properties of mouse liver glutathione S-transferases

Property	Enzyme				
	P1	P2	P3	P4	P5
Elution from Bio-Gel HPHT (min)	31.5	36.0	38.7	44.0	55.0
Isoelectric point	8.6	8.6	N. D.	≧ 9.2	7.8–8.2
Subunit Mr	24500	24500	24500 +24000	26000	27000
Cross-reactivity with antiserum against rat subunits: Yf	+	+	+	−	−
Ya	−	−	−	+	−
Yb_1	−	−	−	−	+
Yb_2	−	−	−	−	−
Yc	−	−	−	−	−

Abbreviation: N. D., not determined.

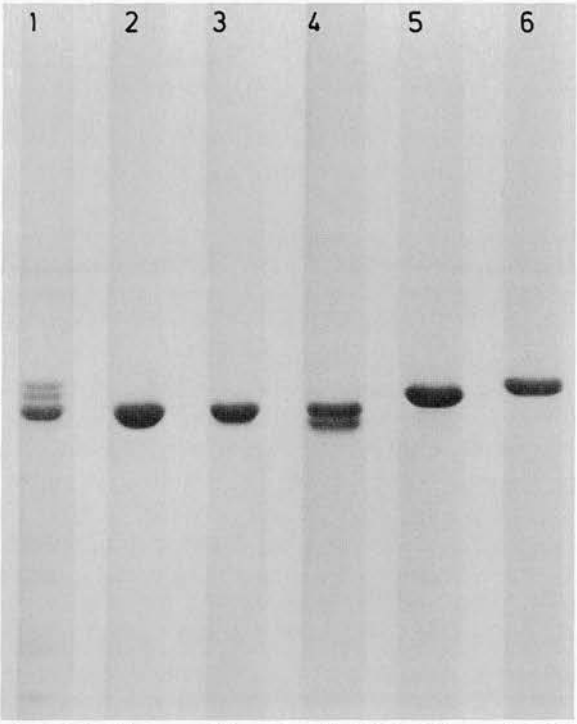

Fig. 4. Subunit composition of purified GST. The peaks of GST that were eluted from the Bio-Gel HPHT column at 31.5, 36.0, 38.7, 44.0 and 55.0 min, designated GST P1–5 respectively, were analysed by SDS/PAGE. The polyacrylamide-gel was loaded as follows: track 1, total hepatic GST; track 2, GST P1; track 3, GST P2; track 4, GST P3; track 5, GST P4; track 6, GST P5

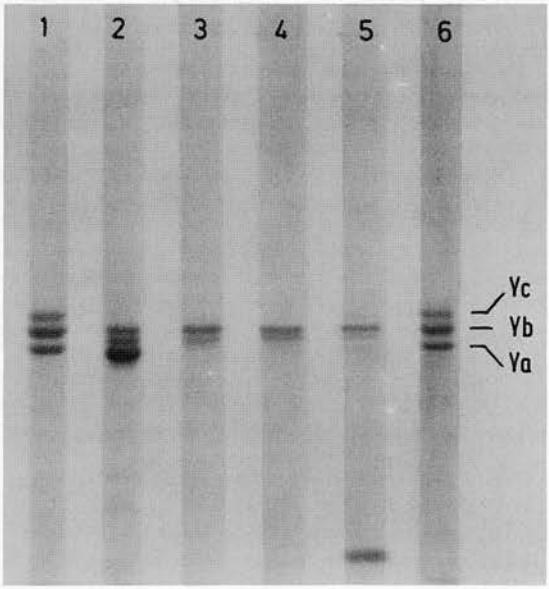

Fig. 5. Tissue distribution of GST subunits. The GST in liver, kidney, testis and lung were purified by S-hexylglutathione-Sepharose 6B chromatography and examined by SDS/PAGE. The samples were applied to the 12.5% (w/v) polyacrylamide-gel as follows: tracks 1 and 6, rat liver GST; track 2, mouse liver GST; track 3, mouse kidney GST; track 4, mouse testis GST; track 5, mouse lung GST. The rat, Ya (Mr 25 500), Yb (Mr 27 000) and Yc (Mr 28 500) subunits are shown

Discussion

Mouse GST transferases have not been intensively studied, unlike the rat GST. The relationship between mouse and rat enzymes is poorly understood at a catalytic level, subunit level and immunochemical level.

In the rat, three multi-gene families of GST exist. Group I comprises Ya-, Yc- and Yk-type subunits; Group II comprises Yb- and Yn-type subunits; Group III comprises Yf-type subunits (Hayes and Mantle 1986). Normal rat liver expresses all subunits except Yf.

In the present study the use of a HPLC hydroxyapatite column (Bio-Gel HPHT) to purify GST from mouse liver has been described. Combined use of S-hexylglutathione-Sepharose 6B and hydroxyapatite HPLC provides very good recovery of GST (>85%) and, certainly in the mouse, excellent resolution. This two-step purification resulted in the isolation of four major peaks of activity, P1, P2, P4 and P5. The P1 and P2 peaks comprise Yf-type subunits (Mr 24500), P4 comprises Ya-type subunits (Mr 26000) and P5 comprises Yb-type subunits (Mr 27000). A further minor enzyme P3 contained Yf-type subunits and a polypeptide (Mr 24000) with a faster electrophoretic mobility than Yf. The GST in the P1, P2, P4 and P5 peaks have isoelectric points of 8.6, 8.6, 9.2 and 7.8–8.2 respectively. The biochemical and immunochemical properties of P1 and P2 are similar, and these two proteins may be coded by the same gene.

The data presented strongly suggest the existence of at least three major genetically distinct GST subunits in mouse liver and an additional minor polypeptide (Mr 24000) that may hybridize with Yf. This minor monomer is also expressed in mouse kidney.

Lee et al. (1981) have previously purified three GST forms from mouse liver, designated F1, F2 and F3. The F1 and F2 forms were immunochemically identical and, as they yielded closely similar peptide maps, it was considered that they might share a common genetic origin. Significant differences exist between the properties of the enzymes purified during the present study, P1–P5, and F1–F3 described by Lee and his colleagues; these prevent unequivocal identification. The fact that Lee et al. (1981) studied DBA/2J mice may suggest differences exist in the GST expressed by different mouse strains.

All the mouse and rat GST subunits that are immunochemically-related were found to possess similar mobilities during SDS/PAGE. This not only greatly facilitated subunit identification but suggests that certain transferases are conserved between species. The N-terminal sequence data of Frey et al. (1983) for the rat Yb subunits, and the deduced sequences of cloned cDNA coding mouse GST (Pearson et al. 1983), support this hypothesis.

Here it has been shown that each of the three multi-gene GST families found in rat and man are also represented in the mouse. In view of the apparent structural homology between the species, it seems surprising that the catalytic activities of the homologous subunits are not highly conserved. For example, the mouse Ya subunit possesses little activity for Δ^5 androstene-3,17-dione (Table 1). By contrast, both mouse and rat Ya subunits are active with cumene hydroperoxide; this may reflect the possibility that stronger selection pressures exist for the glutathione peroxidase activity expressed by GST than their ketosteroidisomerase activity.

Like other mammalian species, the GST in the mouse are subject to tissue-specific expression and different isoenzymes are found in different organs. However, the pattern found in the mouse is distinct. Of the species studied, the mouse was the only species that expressed Yf in the liver. In rat the Yf subunit is only found in the liver in pre-neoplastic lesions and, at a reduced level, in hepatomas. Moreover, in the mouse Yf was essentially absent from extra-hepatic tissues whereas in rat and man this subunit is widely distributed in extra-hepatic tissues. The physiological implications of this finding are unclear but interferon-treatment has been shown to markedly induce the Yf subunit and this finding is being further investigated.

Acknowledgements: Part of this work was funded by a Medical Research Council project grant (G8126 392 SB, awarded to JDH) and a grant from the Cancer Research Advancement Board (awarded to TJM). We thank Mrs E Ward for help in preparing this script.

References

Agius C, Gidari AS (1985) Effect of streptozotocin on the glutathione S-transferases of mouse liver cytosol. Biochem Pharmacol 34:811–819

Boyland E, Chasseaud LF (1969) The role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis. Adv Enzymol 32:173–219

Chasseaud LF (1976) Conjugation with glutathione and mercapturic acid excretion. In: Arias IM, Jakoby WB (eds) Glutathione: Metabolism and Function. Raven Press, New York, pp 77–114

Coles B, Meyer DJ, Ketterer B, Stanton CA, Garner RC (1985) Studies on the detoxication of microsomally-activated aflatoxin B_1 by glutathione and glutathione transferases in vitro. Carcinogenesis 6:693–697

Farber E (1984) Pre-cancerous steps in carcinogenesis: their physiological adaptive nature. Biochim Biophys Acta 738:171–180

Frey AB, Friedberg T, Oesch F, Kreibich G (1983) Studies on the subunit composition of rat liver glutathione S-transferases. J Biol Chem 258:11321–11325

Glatt H, Friedberg T, Grover PL, Sims P, Oesch F (1983) Inactivation of a diol-epoxide and a K-region epoxide with high efficiency by glutathione transferase X. Cancer Res 43:5713–5717

Habig WH, Jakoby WB (1981) Assays for differentiation of glutathione S-transferases. Meth Enzymol 77:398–405

Hayes JD (1984) Purification and characterization of glutathione S-transferases P, S and N. Isolation from rat liver of Yb_1Yn protein, the existence of which was predicted by subunit hybridization in vitro. Biochem J 224:839–852

Hayes JD (1986) Purification and physical characterization of glutathione S-transferase K. Differential use of S-hexyl-glutathione and glutathione affinity matrices to isolate a novel glutathione S-transferase from rat liver. Biochem J 233:789–798

Hayes JD, Strange RC, Percy-Robb IW (1981) A study of the structures of the YaYa and YaYc glutathione S-transferases from rat liver cytosol. Evidence that the Ya monomer is responsible for lithocholate-binding activity. Biochem J 197:491–502

Hayes JD, Chalmers J (1983) Bile acid inhibition of basic and neutral glutathione S-transferases in rat liver. Biochem J 215:581–588

Hayes JD, Mantle TJ (1986) Use of immuno-blot techniques to discriminate between the glutathione S-transferase Yf, Yk, Ya, Yn/Yb and Yc subunits and to study their distribution in extra-hepatic tissues. Evidence for three immunochemically distinct groups of transferase in the rat. Biochem J 233:779–788

Jakobson I, Askelof P, Warholm M, Mannervik B (1977) A steady-state-kinetic random mechanism for glutathione transferase A from rat liver. Eur J Biochem 77:253–262

Jakoby WB (1978) The glutathione S-transferases: A group of multifunctional detoxification proteins. Adv Enzymol 46:383–414

Jensson H, Eriksson LC, Mannervik B (1985) Selective expression of glutathione transferase isoenzymes in chemically-induced pre-neoplastic rat hepatocyte nodules. FEBS Lett 187:115–120

Jernstrom B, Martinez M, Meyer DJ, Ketterer B (1985) Glutathione conjugation of the carcinogenic and mutagenic electrophile (±)-7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetra hydrobenzo(a)pyrene catalyzed by purified rat liver glutathione transferases. Carcinogenesis 6:85–89

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227:680–685

Lee C-Y, Johnson L, Cox RH, McKinney JD, Lee S-M (1981) Mouse liver glutathione S-transferases. Biochemical and immunochemical characterization. J Biol Chem 256:8110–8116

Lee C-YG (1982) Biochemical and immunochemical analysis of an abundant form of glutathione S-transferase in mouse testis. Mol Cell Biochem 49:161–168

Mannervik B (1985) The isoenzymes of glutathione transferase. Adv Enzymol 57:357–417

Mannervik B, Guthenberg C (1981) Glutathione transferase (human placenta). Meth Enzymol 77:231–235

Meyer DJ, Beale D, Tan KH, Coles B, Ketterer B (1985) Glutathione transferases in primary rat hepatomas: the isolation of a form with GSH peroxidase activity. FEBS Lett 184:139–143

Pearson WR, Windle JJ, Morrow JF, Benson AM, Talalay P (1983) Increased synthesis of glutathione S-transferases in response to anticarcinogenic antioxidants. Cloning and measurement of messenger RNA. J Biol Chem 258:2052–2062

Satoh K, Kitahara A, Soma Y, Inaba Y, Hatayama I, Sato K (1985) Purification, induction and distribution of placental glutathione transferase. A new marker enzyme for preneoplastic cells in the rat chemical hepatocarcinogenesis. Proc Natl Acad Sci USA 82:3964–3968

Simons PC, Vander Jagt DL (1977) Purification of glutathione S-transferases from human liver by glutathione-affinity chromatography. Anal Biochem 82:334–341

Smith GJ, Ohl VS, Litwack G (1977) Ligandin, the glutathione S-transferases and chemically-induced hepatocarcinogenesis: a review. Cancer Res 37:8–14

Sparnins VL, Venegas PL, Wattenberg LW (1982) Glutathione S-transferase activity: enhancement by compounds inhibiting chemical carcinogenesis and by dietary constituents. J Natl Cancer Inst 68:493–496

Towbin H, Staehlin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

The studies from the authors' laboratory were supported by grants from the Swedish Natural Science Research Council, the Swedish Cancer Society and from the National Cancer Institute, N.I.H., D.H.E.W., Bethesda, Maryland, U.S.A.

Botti, B., Moslen, M. T., Trieff, N. M. & Reynolds, E. S. (1982) *Chem.-Biol. Interact.* **42**, 259–270
Boyer, T. D., Zakim, D. & Vessey, D. A. (1983) *Biochem. Pharmacol.* **32**, 29–35
Burke, R. F. (1983) *Biochim. Biophys. Acta* **757**, 21–28
Chasseaud, L. F. (1979) *Adv. Cancer Res.* **29**, 175–274
DePierre, J. W. & Dallner, G. (1975) *Biochim. Biophys. Acta* **415**, 411–472
Dohn, D. R. & Anders, M. W. (1982) *Biochem. Biophys. Res. Commun.* **109**, 1339–1345
Friedberg, T., Bentley, P., Stasiecki, P., Glatt, H. R., Raphael, D. & Oesch, F. (1979) *J. Biol. Chem.* **254**, 12028–12033
Jacoby, W. B. & Habig, W. H. (1980) in *Enzymatic Basis of Detoxication* (Jacoby, W. B., ed.), vol. 2, pp. 63–94, Academic Press, New York
Jensson, H., Guthenberg, C., Alin, P. & Mannervik, B. (1986) *FEBS Lett.* **203**, 207–209
Kraus, P. (1975) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **291**, 79–87
Lee, C. G. & McKinney, J. D. (1982) *Mol. Cell Biochem.* **48**, 91–96
Mannervik, B. (1985) *Adv. Enzymol. Relat. Areas Mol. Biol.* **57**, 357–417
Masukawa, T. & Iwata, H. (1986) *Biochem. Pharmacol.* **35**, 435–438
Morgenstern, R. & DePierre, J. W. (1983) *Eur. J. Biochem.* **134**, 591–597
Morgenstern, R. & DePierre, J. W. (1985) in *Reviews in Biochemical Toxicology* (Hodgson, E., Bend, J. R. & Philpot, R. M., eds.), pp. 67–104, vol. 7, Elsevier, New York
Morgenstern, R., DePierre, J. W. & Ernster, L. (1979) *Biochem. Biophys. Res. Commun.* **87**, 657–663
Morgenstern, R., Guthenberg, C. & DePierre, J. W. (1982*a*) *Eur. J. Biochem.* **28**, 243–248
Morgenstern, R., Guthenberg, C., Mannervik, B. & DePierre, J. W. (1982*b*) *Cancer Res.* **42**, 4215–4222
Morgenstern, R., Guthenberg, C., Mannervik, B. & DePierre, J. W. (1983) *FEBS Lett.* **160**, 264–268
Morgenstern, R., Lundqvist, G., Andersson, G., Balk, L. & DePierre, J. W. (1984) *Biochem. Pharmacol.* **33**, 3609–3614
Morgenstern, R., DePierre, J. W. & Jörnvall, H. (1985) *J. Biol. Chem.* **260**, 13976–13983
Odum, J. & Green, T. (1984) *Toxicol. Appl. Pharmacol.* **76**, 306–312
Prohaska, J. R. (1980) *Biochim. Biophys. Acta* **611**, 87–98
Sawahata, T. & Neal, R. A. (1983) *Mol. Pharmacol.* **23**, 453–460
Tan, K. H., Meyer, D. J., Belin, J. & Ketterer, B. (1984) *Biochem. J.* **220**, 243–252
Wolf, C. R., Berry, P. N., Nash, J. A., Green, T. & Lock, E. A. (1984) *J. Pharmacol. Exp. Therm.* **228**, 202–210

Received 8 January 1987

Glutathione *S*-transferases in man: the relationship between rat and human enzymes

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Glutathione *S*-transferase (GST) is an important member of the phase II detoxification enzyme system; GST activity is responsible for catalysing the conjugation of glutathione to a large spectrum of lipophilic electrophiles (Jakoby, 1978; Chasseuad, 1979). This reaction is the first step in the formation of mercapturic acids, a pathway that provides an important means of eliminating compounds that are potentially cytotoxic or mutagenic from the body. Like other drug-metabolizing enzymes, GST activity can be induced by the administration of foreign compounds such as phenobarbital and 3-methylcholanthrene, or by the ingestion of food additives like butylated hydroxyanisole (Benson *et al.*, 1978; Hayes *et al.*, 1979; Ding *et al.*, 1986). The effects of xenobiotics have been studied primarily in the liver, but intestinal and renal GST activity can also be induced (Hales & Neims, 1977; Clifton & Kaplowitz, 1978). Increased intracellular levels of transferase have been reported to protect rodents against carcinogenesis (Wattenburg, 1983); this induction process appears to be important in the mode of action of anticarcinogenic agents such as benzyl isothiocyanate, β-naphthoflavone, coumarin, disulphiram and indole-3-acetonitrile (Sparnins *et al.*, 1982).

The GST are widely distributed in the animal kingdom and, in the majority of species studied to date, GST activity has been found to be represented by multiple enzyme forms (Mannervik, 1985). Although only rigorously studied in the rat, the different enzyme forms that have been isolated from various species are considered to be distinct isoenzymes that are coded for by multiple genes (Lai *et al.*, 1984; Pickett *et al.*, 1984; Tu *et al.*, 1984; Ding *et al.*, 1985; Morgenstern *et al.*, 1985; Sugoka *et al.*, Alin *et al.*, 1986).

Abbreviations used: GST, glutathione *S*-transferase; PAGE, polyacrylamide-gel electrophoresis.

The GST have been referred to as 'multi-functional' proteins (Jakoby & Keen, 1977) and there is much speculation about their various physiological roles (Kaplowitz, 1980; Kefferer, 1982; Habig, 1983; Mannervik, 1985). The fact that these enzymes represent as much as 4% of the cytosolic protein in the liver, and 1% of the protein in kidney and intestine, indicates that they play important 'house-keeping' functions in cells. Further, the different GSH isoenzymes appear to have distinct functions as they exhibit different catalytic activities (Hayes, 1984, 1986; Mannervik, 1985) and their expression is organ-specific (Hayes & Mantle, 1986*a*). Differences in the location of the isoenzymes within tissues have been noted (Redick *et al.*, 1982) as well as differences in the subcellular localization of individual GST (Campbell *et al.*, 1980; Bennett *et al.*, 1986).

The variations in the distribution of GST, both between and within organs, suggest that complex regulatory mechanisms controlling their expression exist. Remarkably little is known of the biological control of GST (for a review, Mannervik, 1985). The levels of hepatic GST increase rapidly postnatally and, in the rat, reach adult levels after about 7 weeks (Hales & Neims, 1976*b*). Sex differences in both hepatic and renal GST activities have been described (Darby & Grundy, 1982) and a limited literature exists describing the hypothalamic and pituitary modulation of the gonadal control of GST in rat liver (Hales & Neims, 1976*a*; Lamartinier, 1981). Recently, interest has focused on placental GST since it is markedly induced in preneoplastic hepatocytes and can serve as a tumour marker (Satoh *et al.*, 1985). The placental isoenzyme may also be partly responsible for the acquired resistance of certain tumours towards cytotoxic drugs.

Enzyme purification

Many different purification strategies have been developed for the cytosolic GST. Affinity chromatography is the method of central importance in the isolation of the soluble enzymes; most methods employ either glutathione–Sepharose or *S*-hexylglutathione–Sepharose as the matrix to isolate GST (Simmons & Vander Jagt, 1977; Mannervik

& Guthenberg, 1981). As currently used, these affinity gels result in the recovery of GST as a single pool of activity (cf. Fig. 2 discussed below), and chromatofocusing or hydroxyapatite chromatography are often used to resolve the individual enzymes.

Purification of microsomal GST has been developed by Morgenstern *et al.* (1982). These workers found that, by contrast with cytosolic GST enzymes, the activity of microsomal GST is markedly increased by treatment with *N*-ethylmaleimide. The purification method devised for microsomal GST involves activation with *N*-ethylameimide, solubilization with Triton X-100, and sequential elution from columns of hydroxyapatite and CM-Sepharose (see also Morgenstern & DePierre, 1987).

Rat GST

In the rat, both cytosolic and microsomal GST have been purified. The cytosolic forms have native M_r of 48 000–55 000, and comprise two subunits. Collectively, these soluble enzymes contain six subunit types, designated Ya (M_r 25 500), Yb (M_r 26 300), Yc (M_r 27 500), Yf (M_r 24 800), Yk (M_r 25 000) and Yn (M_r 26 000) (Hayes, 1984, 1986; Hayes & Mantle, 1986*b*); the Yf-type polypeptide has also been referred to as Yp since it represents the major placental GST subunit (Kitahara *et al.*, 1984). An additional lower-M_r GST polypeptide, preliminarily designated Ye (M_r 24 200), has been identified but its relationship to Yf awaits clarification (Hayes & Mantle, 1986*b*).

Heterodimeric and homodimeric combinations of these subunits occur, but only polypeptides with extensive sequence homology (>65%) can hybridize (Boyer *et al.*, 1983; Hayes, 1983, 1984); the cytosolic subunits all possess a minimum of about 25% homology with each other (see also Tu & Qian, 1987). By contrast, microsomal GST has a calculated M_r, when solubilized with Triton X-100, of 127 000 and is composed of three identical subunits (Morgenstein *et al.*, 1985). The microsomal GST polypeptide is unique, has an M_r of 17 000 and does not appear to combine with the cytosolic GST subunits.

From the available data, both immunochemical and molecular, rat GST can be divided in four classes. Group I contains the cytosolic Ya-, Yc- and Yk-type subunits; group II contains the cytosolic Yb- and Yn-type subunits; group III comprises the Yf- and possibly the Ye-) type polypeptides; the microsomal enzyme is a member of a separate GST class. Little is known of the genomic structures and chromosomal locations of the genes encoding these subunits (Rothkopf *et al.*, 1986). Whilst it will be desirable eventually to have defined the GST by molecular genetic criteria according to the chromosomal locations of their gene clusters, it is unclear at present what the molecular significance is of dividing the GST enzymes into the four classes outlined above. For example, although the Ya- and Yc-type subunits possess about 70% sequence homology, and have been allocated to the same GST class, Li *et al.* (1986) suggested (on the basis of organ distribution and their differential phenobarbital inducibility) that the Ya and Yc genes are members of independent gene families.

Human GST

Subunit structure of cytosolic and microsomal enzymes. The cytosolic GST enzymes in man have been divided, on the basis of their charge, into 'basic', 'neutral' (or 'near-neutral') and 'acidic' forms. (Warholm *et al.*, 1983; Singh *et al.*, 1985; Stockman *et al.*, 1985; Vander Jagt *et al.*, 1985; Soma *et al.*, 1986). This type of classification is commonly used but may be misleading in certain circumstances since the distribution between the groups is not clear-cut. Particular difficult can be experienced in identifying GST of pI 5.0–5.5 or pI 6.7–7.3. In addition, certain 'basic' human GST may be mis-classified if designations are made on the basis of chromatographic properties, since at least one of the isoenzymes is strongly hydrophobic and, as a result of interactions with column matrices, may elute with 'acidic' and 'neutral' GST (Hayes *et al.*, 1983; Soma *et al.*, 1986).

A more reliable grouping of these enzymes can be achieved on the basis of the migration of their constituent subunits during SDS PAGE. When SDS PAGE that employs a 12% (w/v) polyacrylamide resolving gel and incorporates 0.32% (w/v) *N*,*N*′-methylene-bisacrylamide is used, the polypeptides that make up the human cytosolic GST have apparent M_r of 24 800, 26 000 and 26 700; these correspond to the major 'acidic', 'basic' and 'neutral' GST respectively. By probing these polypeptides with antisera raised against individual rat GST, immunoblots have shown that the human M_r 24 800, 26 000 and 26 700 polypeptides are immunochemically related to rat Yf, Ya and Yb_1 subunits, respectively (Hayes & Mantle, 1986*a*,*b*); no cross-reactivity was observed between human GST and antiserum raised against the rat Yc subunit.

It is apparent that the human enzymes which have been referred to as 'basic', 'neutral' and 'acidic' GST are composed of Ya-, and Yb- and Yf-type subunits, respectively. However, it should be noted that some other workers have simply divided the GST into anionic and cationic forms (Awasthi *et al.*, 1980; Pattinson, 1981; Koskelo, 1983); in these instances it is likely the cationic forms comprise Ya-type subunits but, unless the enzymes have been analysed by SDS PAGE and the system employed is clearly defined, it is unclear whether the anionic forms represent Yb- or Yf-type polypeptides.

Electrophoretic results from different groups must be treated with some caution (e.g. Stockman *et al.*, 1985; Vander Jagt *et al.*, 1985; Soma *et al.*, 1981) since the relative mobilities of GST polypeptides during SDS/PAGE vary according to the amount of *N*,*N*′-methylene-bisacrylamide cross-linker employed in the resolving gel (Hayes & Mantle, 1986*b*). In resolving gels which contain normal levels of cross-linker (C_{Bis} 2.6%) the order of mobility of the rat subunits is Yf > Ya > Yb > Yc (Fig. 1). By contrast, at low degrees of cross-linking (C_{Bis} 0.6%) the order of migration of rat subunits is Ya > Yf > Yb > Yc. The human GST subunits behave in accord with their rat counterparts.

The change in migration order of GST polypeptides is due to the anomalous electrophoretic behaviour of the rat Ya and human 'basic' GST subunits; the relative positions of the other polypeptides shown in Fig. 1 do not appear to change markedly with different degrees of cross-linking. It has been suggested that the variable mobility, which both the rat Ya and human 'basic' GST subunits display, is due to hydrodynamic effects arising from the fact that these polypeptides possess blocked *N*-termini (Hayes & Mantle, 1986*b*).

When antiserum raised against the rat microsomal GST is used to probe human liver microsomal protein resolved by SDS PAGE, the immune replicas show the existence of an immunoreactive polypeptide, with the same apparent mobility (M_r 17 000) as rat microsomal GST, in the human sample. The GST in human microsomes can be isolated using methods similar to those devised by Morgenstern *et al.* (1985) for the rat enzyme. The purified human enzyme displays a strong immunochemical cross-reactivity with the rat GST (L. I. McLellan & J. D. Hayes, unpublished work).

Polymorphism. Although microsomal GST appears to be expressed in all individuals (L. I. McLellan & J. D. Hayes, unpublished work), significant variations exist in the cytosolic GST content of different livers. A starch-gel zymogram method has been used to show that inter-individual differences occur in the charge of hepatic GST (Board, 1981;

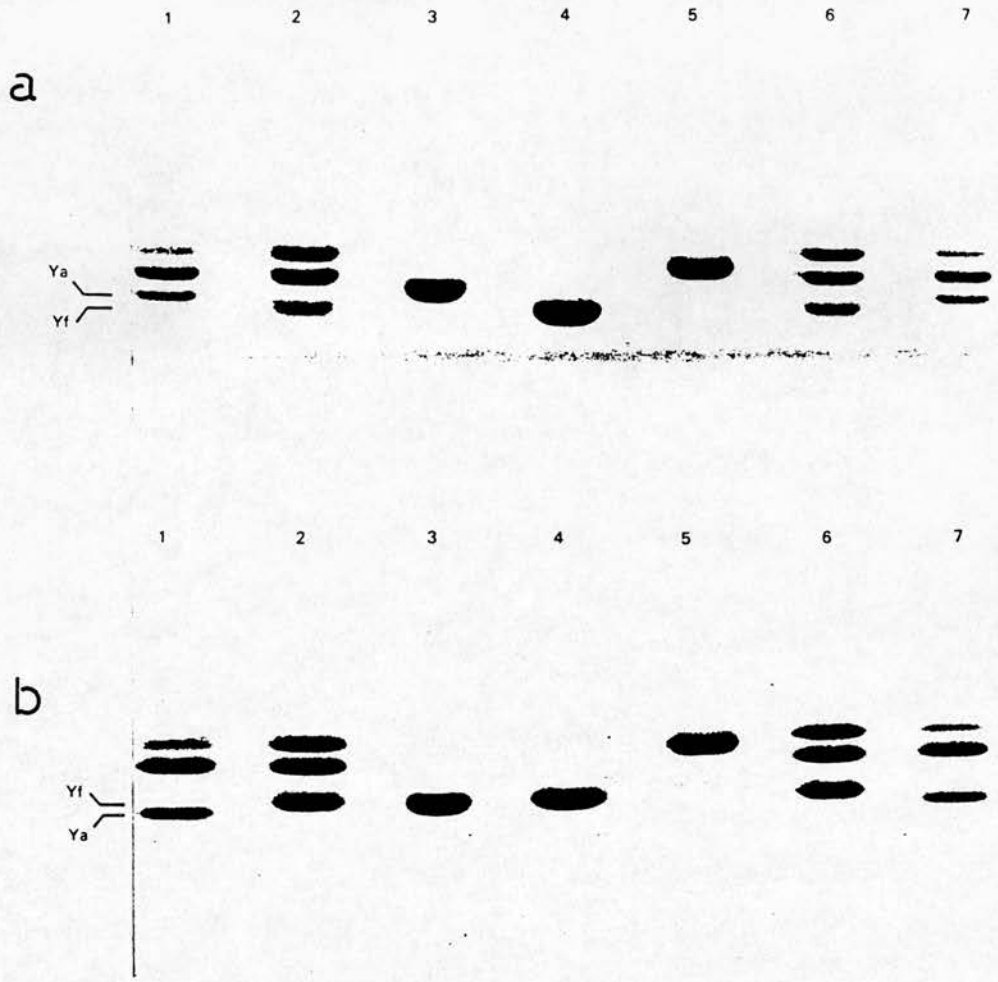

Fig. 1. *Variable electrophoretic mobility of Ya-type subunits isolated from rat and man*

SDS/PAGE was performed in resolving gels that comprised (*a*) 12% (w/v) polyacrylamide containing 0.32% (w/v) *N*,*N*′-methylene-bisacrylamide or (*b*(15% (w/v) polyacrylamide containing 0.08% (w/v) *N*,*N*′-methylene-bisacrylamide (Hayes & Mantle, 1986*b*). The same protein samples were applied in both panels as follows: tracks 1 and 7, rat liver GST (Ya, Yb, Yc); tracks 2 and 6, rat lung GST (Yf, Yb, Yc); track 3, human 'basic' GST γ (B_2B_2); track 4, human 'acidic' GST λ; track 5, human 'neutral' GST μ. The rat Ya and Yf subunits are indicated.

Strange *et al.*, 1984). Board (1981) proposed that the patterns of activity observed by the zymogram technique had a genetic basis and were attributable to three loci, called GST 1, GST 2 and GST 3. It is probable the three GST loci encode the Yb-, Ya- and Yf-type subunits, respectively (Hussey *et al.*, 1986; Tu *et al.*, 1986).

A common 'null' phenotype exists at the GST 1 locus and it has been recognized for some time that the Yb-containing enzyme, GST μ, is only expressed in about 60% of liver specimens (Warholm *et al.*, 1983). At a molecular level, it is not known why the Yb-polypeptide fails to be expressed in the remaining 40% of human livers.

Board (1981) postulated that at least two autosomal alleles exist at the GST 2 locus, and allelic variation can account for the phenotypes observed (cf. Strange *et al.*, 1984). It has subsequently been demonstrated that two distinct Ya-type subunits are present in the population; these can be isolated from human liver as homodimers or a heterodimer (Stockman *et al.*, 1985).

Variations in the cytosolic GST content of different liver specimens can be so great that it is advisable to perform an initial 'screening' of livers before undertaking a large-scale enzyme purification (Hussey *et al.*, 1986). Human 'basic' Ya-containing GST and 'neutral' Yb-containing GST possess different binding properties for *S*-hexylglutathione–Sepharose 6B, and are eluted respectively from the affinity matrix by concentrations of about 0.03 mmol/l (fractions 15–33) and 0.20 mmol/l (fractions 75–84) *S*-hexylglutathione (Fig. 2). This technique can be used as a simple one-step screening procedure, and should save considerable time during the work-up of suitable livers to allow the study of the 'neutral' Yb-containing enzymes that are coded at the GST 1 locus.

The GST 3 locus is only weakly expressed in normal adult liver, but the Yf-containing GST is present in high levels in fetal liver (Guthenberg *et al.*, 1986; Hussey *et al.*, 1986; Soma *et al.*, 1986). The Yf-type GST subunit is found in placenta and adult human lung (Dao *et al.*, 1984) as well as in erythrocytes (Vander Jagt *et al.*, 1985). Guthenberg *et al.* (1986) showed that the fetal Yf-containing GST is cata-

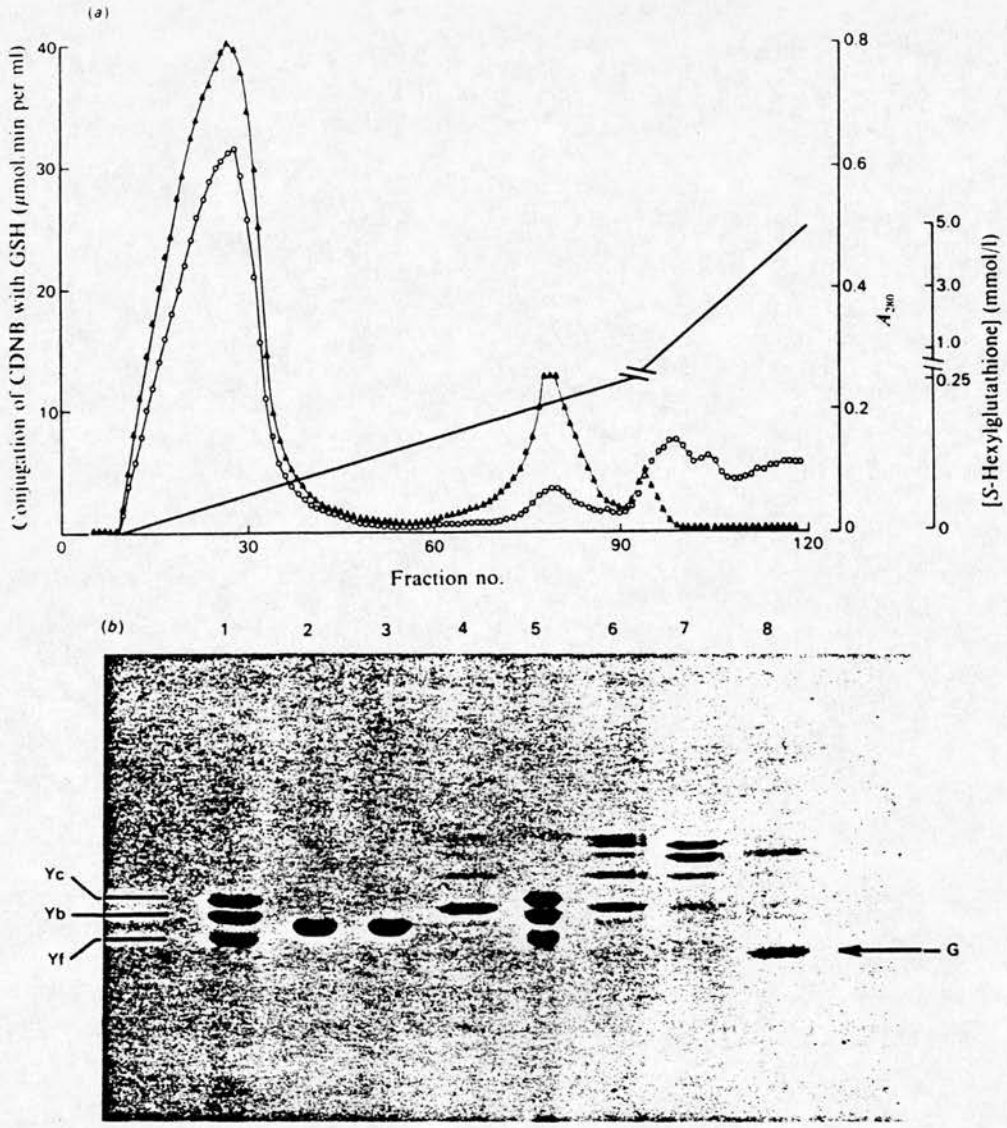

Fig. 2. *Gradient elution of human liver GST from S-hexylglutathione–Sepharose 6B*

(*a*) Human liver cytosol (180 ml, 6 g of protein) was applied to a 1.6 cm × 16.0 cm column of *S*-hexylgutathione–Sepharose 6B. The affinity matrix was washed (24 ml/h) with about 500 ml fo 40 mM-Tris/HCl buffer (pH 7.8) that contained 200 mM-NaCl before the column was developed with a continuous gradient of *S*-hexylgultathione that was formed in two separate stages (i.e. 0–0.25 mmol/l over 400 ml followed, sequentially, by 0.25–5.0 mmol/l over 150 ml); the gradient is represented by a solid line. Fractions of 4.8 ml were collected and GST activity with 1-chloro-2,4-dinitrobenzene (CDNB, ▲) as well as absorbance at 280 nm (○) were measured. (*b*) The polypeptide content of fractions 13, 24, 79, 94, 99 and 104 was determined by SDS/PAGE (in a 12%, w/v, polyacrylamide-gel containing 0.32% cross-linker). The samples loaded (2–5 μg) on to the gel shown were as follows: tracks 1 and 5, rat lung GST (comprising Yf, Yb and Yc); track 2, fraction 13; track 3, fraction 34; track 4, fraction 79; track 6, fraction 94; track 7, fraction 99; track 8, fraction 104. The rat Yf, Yb and Yc subunits are shown and the position of glyoxalase (G) is indicated.

lytically distinct from its counterpart in placenta, but the structural basis for the reported differences requires further study.

Conclusion

The genetic variation associated with GST may be an important factor in determining the susceptibility of different individuals to carcinogens and environmental toxins. It may also be a factor in determining whether certain tumours are amenable to treatment by cytotoxic chemotherapeutic agents.

Work by J.D.H. and colleagues described in this overview was carried out with financial support from the Medical Research Council (project grant G812392SB), and this is gratefully acknowledged. Professor L. G. Whitby is thanked both for critically reading this script and for his continued support. We are grateful to Mrs E. Ward for her secretarial assistance.

Alin, P., Mannervik, B. & Jornvall, H. (1986) *Eur. J. Biochem.* **156**, 343–350
Awasthi, Y. C., Dao, D. D. & Saneto, R. P. (1980) *Biochem. J.* **191**, 1–10
Bennett, C. F., Spector, D. L. & Yeoman, L. C. (1986) *J. Cell Biol.* **102**, 600–609
Benson, A. M., Batzinger, R. P., Ou, S.-Y. L., Bueding, E., Cha, Y.-N. & Talalay, P. (1978) *Cancer Res.* **38**, 4486–4495
Board, P. G. (1981) *Am. J. Hum. Genet.* **33**, 36–43
Boyer, T. D., Kenney, W. C. & Zakim, D. (1983) *Biochem. Pharmacol.* **32**, 1843–1850
Campbell, J. A. H., Bass, N. M. & Kirsch, R. E. (1980) *Cancer* **45**, 503–510
Chasseaud, L. F. (1979) *Adv. Cancer Res.* **29**, 175–274
Clifton, G. & Kaplowitz, N. (1978) *Biochem. Pharmacol.* **27**, 1284–1287
Clifton, G., Kaplowitz, N., Wallin, J. D. & Kuhlenkamp, J. (1975) *Biochem. J.* **150**, 259–262
Dao, D. D., Partridge, C. A., Kurosky, A. & Awasthi, Y. C. (1984) *Biochem. J.* **221**, 33–41
Darby, G. J.-F., Lu, A. Y. H. & Pickett, C. B. (1985) *J. Biol. Chem.* **260**, 13268–13271
Ding, G. J.-F., Ding, V. D.-H., Rodkey, J. A., Bennett, C. D., Lu, A. Y. H. & Pickett, C. B. (1986) *J. Biol. Chem.* **261**, 7952–7957
Guthenberg, C., Warholm, M., Rane, A. & Mannervik, B. (1986) *Biochem. J.* **235**, 741–745
Habig, W. H. (1983) in *Radioprotectors and Anticarcinogens* (Nygaard, O. F. & Simic, M. G., eds.), pp. 169–190, Academic Press, New York
Hales, B. F. & Neims, A. H. (1976*a*) *Biochem. J.* **160**, 223–229
Hales, B. F. & Neims, A. H. (1976*b*) *Biochem. J.* **160**, 231–236
Hales, B. F. & Neims, A. H. (1977) *Biochem. Pharmacol.* **26**, 555–556
Hayes, J. D. (1983) *Biochem. J.* **213**, 625–633
Hayes, J. D. (1984) *Biochem. J.* **224**, 839–852
Hayes, J. D. (1986) *Biochem. J.* **233**, 789–798
Hayes, J. D. & Mantle, T. J. (1986*a*) *Biochem. J.* **233**, 779–788
Hayes, J. D. & Mantle, T. J. (1986*b*) *Biochem. J.* **237**, 731–740
Hayes, J. D., Strange, R. C. & Percy-Robb, I. W. (1979) *Biochem. J.* **181**, 699–708
Hayes, J. D., Gilligan, D., Chapman, B. J. & Beckett, G. J. (1983) *Clin. Chim. Acta* **134**, 107–121
Hussey, A. J., Stockman, P. K., Beckett, G. J. & Hayes, J. D. (1986) *Biochim. Biophys. Acta* **874**, 1–12
Jakoby, W. B. (1978) *Adv. Enzymol. Relat. Areas mol. Biol.* **46**, 383–414
Jakoby, W. B. & Keen, J. H. (1977) *Trends Biochem. Sci.* **2**, 229–231
Kaplowitz, N. (1980) *Am. J. Physiol.* **239**, G439–G444
Kaplowitz, N., Kuhlenkamp, J. & Clifton, G. (1975) *Biochem. J.* **146**, 351–356
Ketterer, B. (1982) *Drug Metab. Rev.* **13**, 161–187
Kitahara, A., Satoh, K., Nishimura, K., Ishikawa, T., Ruike, K., Sato, K., Tsuda, H. & Ito, N. (1984) *Cancer Res.* **44**, 2698–2703
Koskelo, K. (1983) *Scand. J. Clin. Lab. Invest.* **43**, 133–139
Lai, H.-C. J., Li, N., Weiss, M. J., Reddy, C. C. & Tu, C.-P. D. (1984) *J. Biol. Chem.* **259**, 5536–5542
Lamartiniere, C. A. (1981) *Biochem. J.* **198**, 211–217
Li, N.-q., Reddanna, P., Thyagaraju, K., Reddy, C. C. & Tu, C.-P. D. (1986) *J. Biol. Chem.* **261**, 7596–7599
Mannervik, B. (1985) *Adv. Enzymol. Relat. Areas Mol. Biol.* **57**, 357–417
Mannervik, B. & Guthenberg, C. (1981) *Methods Enzymol.* **77**, 231–235 719–721
Morgenstern, R. & De Pierre (1987) *Biochem. Soc. Trans.* **15**, 000–000
Morgenstern, R., Guthernberg, C. & De Pierre, J. W. (1982) *Eur. J. Biochem.* **128**, 243–248
Morgenstern, R., De Pierre, J. W. & Jornvall, H. (1985) *J. Biol. Chem.* **260**, 13976–13983
Pattinson, N. R. (1981) *Biochem. Biophys. Res. Commun.* **102**, 403–410
Pickett, C. B., Telakowski-Hopkins, C. A., Ding, G. J.-F., Argenbright, L. & Lu, A. Y. H. (1984) *J. Biol. Chem.* **259**, 5182–5188
Redick, J. A., Jakoby, W. B. & Baron, J. (1982) *J. Biol. Chem.* **257**, 15200–15203
Rothkopf, G. S., Telakowski-Hopkins, C. A., Stotish, R. L. & Pickett, C. B. (1986) *Biochemistry* **25**, 993–1002
Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. & Sato, K. (1985) *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 3964–3968
Simons, P. C. & Vander Jagt, D. L. (1977) *Anal. Biochem.* **82**, 334–341
Singh, S. V., Dao, D. D., Partridge, C. A., Theodore, C., Srivastava, S. K. & Awasthi, Y. C. (1985) *Biochem. J.* **232**, 781–790
Sparnins, V. L., Venegas, P. L. & Wattenberg, L. W. (1982) *J. Natl. Cancer Res.* **68**, 493–496
Soma, Y., Satoh, K. & Sato, K. (1986) *Biochim. Biophys. Acta* **869**, 247–258
Strange, R. C., Faulder, C. G., Davis, B. A., Hume, R., Brown, J. A. H., Cotton, W. & Hopkinson, D. A. (1984) *Ann. Hum. Genet.* **48**, 11–20
Stockman, P. K., Beckett, G. J. & Hayes, J. D. (1985) *Biochem. J.* **227**, 457–465
Suguoka, Y., Kano, T., Okuda, A., Sakia, M., Kitagawa, T. & Maramatsu, M. (1985) *Nucleic Acids Res.* **13**, 6049–6057 734–736
Tu, C.-P. D. & Qian, B. (1987) *Biochem. Soc. Trans.* **15**, 000–000
Tu, C.-P. D., Lai, H.-C. J., Li, N., Weiss, M. J. & Reddy, C. C. (1984) *J. Biol. Chem.* **259**, 9434–9439
Tu, C.-P. D., Matsushima, A., Li, N.-q., Rhoads, D. M., Srikumar, K., Reddy, A. P. & Reddy, C. C. (1986) *J. Biol. Chem.* **261**, 9540–9545
Vander Jagt, D. L., Hunsaker, L. A., Garcia, K. B. & Royer, R. E. (1985) *J. Biol. Chem.* **260**, 11603–11610
Warholm, M., Guthenberg, C. & Mannervik, B. (1983) *Biochemistry* **22**, 3610–3617
Wattenberg, L. W. (1983) *Cancer Res.* **43** (Suppl.), 2448s–2453s

Received 8 January 1987

Expression of genes encoding glutathione *S*-transferases in normal and preneoplastic liver

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The glutathione *S*-transferases are comprised of a family of proteins that catalyse the conjugation of glutathione to various electrophilic substrates. In addition to the conjugation activity, the transferases bind a number of hydrophobic compounds such as haem, bilirubin, polycyclic aromatic hydrocarbons and dexamethasone (Arias *et al.*, 1976; Litwack *et al.*, 1971; Jakoby & Habig, 1980). The glutathione *S*-transferases are heterodimers or homodimers comprised of at least seven subunits (Bass *et al.*, 1977). Many of the glutathione *S*-transferases are induced by various xenobiotics (e.g. phenobarbital and 3-methylcholanthrene) and are elevated in persistent hepatocyte nodules induced by chemical carcinogens.

In this paper, we would like to summarize data from our laboratory that addresses how the level of the glutathione *S*-transferases are regulated by xenobiotics and during chemical carcinogenesis. We will present evidence that indicates the genes encoding the glutathione *S*-transferases are transcriptionally activated by xenobiotics, which leads to an elevation in specific glutathione *S*-transferase mRNAs in the liver. We will also discuss the expression of genes encoding glutathione *S*-transferase and other phase II drug-metabolizing enzymes during chemical carcinogenesis, and contrast their expression during this process with their expression during xenobiotic administration.

Materials and methods

RNA blots and nuclear run-on assay. Poly(A$^+$)-RNA was

Abbreviation used: TCCD.

HUMAN GLUTATHIONE S-TRANSFERASES

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PREPRINT

SYNOPSIS

Four distinct groups of glutathione S-transferase (GST) have been identified in man; one form, found in human microsomes, is composed of 14.0 kDa subunits whereas the three remaining classes are cytosolic, each comprising distinct subunits of 24.8 kDa (Yf-type), 26.0 kDa (Ya-type) or 26.7 kDa (Yb-type). Purification schemes for the cytosolic GST are described. The protein containing the 24.8 kDa subunit, GST λ, has been isolated from human lung; it has a pI value of 4.8. The isoenzymes that contain 26.0 kDa polypeptides (GST γ, δ and ε), and those containing 26.7 kDa polypeptides (GST μ and ψ), have been purified from hepatic cytosol. Individual hepatic isoenzymes can be identified by their isoelectric points; GST γ, δ and ε have pI values of 8.4, 8.75 and 8.9 whereas GST μ and ψ have pI values of 6.1 and 5.5 respectively. Data presented describing the polymorphism associated with the hepatic enzymes clearly illustrate the advantage of screening a number of livers before undertaking a GST purification. Further, these results suggest that differences may exist between individuals in their ability to detoxify electrophiles via the mercapturic acid excretory pathway.

INTRODUCTION

The glutathione S-transferases (GST) play a pivotal role in drug-metabolism. These enzymes catalyse the formation of a thioether bond between reduced glutathione (GSH) and a large number of lipophilic compounds that possess an electrophilic centre; this conjugation reaction is the first step in the synthesis of mercapturic acids (Jakoby, 1978; Chasseaud, 1979). Some of the compounds that serve as the second substrate for GST, such as halogenated hydrocarbons, may already possess an electrophilic centre and therefore require no modification prior to conjugation with GSH. Other potential substrates may require activation by the cytochrome P450-dependent mono-oxygenases before they can be metabolised by GST. Although primarily thought to provide protection against xenobiotics (Smith et al., 1977; Sparnins et al., 1982; Glatt et al., 1983), it should be noted that the conjugation of GSH by GST to certain foreign compounds may markedly increase their toxicity (Rannug et al., 1978; Wolf et al., 1984).

GST are widely distributed in the animal kingdom and the cytosolic forms that have been isolated from mammalian sources are encoded by three multi-gene families (Mannervik et al., 1985; Hayes & Mantle, 1986a). The cytosolic GST each comprise two subunits of 24.0 - 27.5 kDa. Both homodimers and heterodimers exist but only subunits that are members of the same family can hybridize (Hayes 1984, 1986; Stockman et al., 1985). An additional unique GST, composed of 14.0 kDa subunits, the activity of which is induced by N-ethylmaleimide, has been described in rodents but not in man (Morgenstern et al., 1982, 1984). Each

GST is active towards a distinct spectrum of electrophiles and the individual isoenzymes appear to serve different detoxification roles (Glatt et al., 1983; Coles et al., 1985; Jernstrom et al., 1985).

Recent work has focussed on the catalytic and molecular differences between the various rat GST forms but much less is known about the enzymes in man. Human cytosolic GST have been divided into three groups - 'basic', 'neutral' and 'acidic' (Mannervik, 1985). However, the structural and immunochemical properties of the GST in these groups is confused (cf Awasthi et al., 1980; Mannervik et al., 1985; Singh et al., 1985; Vander Jagt et al., 1985). Furthermore, variations in the GST content of individual human livers have been reported, but the polypeptide basis of these differences is poorly understood (Warholm et al., 1980; Board 1981).

During the present study the polymorphism associated with human GST has been investigated and the properties of the purified enzymes are described. The relationship has been explored between human GST, rat microsomal GST and rat cytosolic Ya (25.5 kDa), Yb_1 (26.3 kDa), Yb_2 (26.3 kDa), Yc (27.5 kDa) and Yf (24.8 kDa) GST subunits (Hayes & Mantle, 1986b).

METHODS

Enzyme assays

These were performed as described by Habig & Jakoby (1981).

Tissue and preparation of extracts

Human livers were obtained within 2 h of death and were stored at -85°C until use. Human lung, that had no macroscopic evidence of disease, was obtained from a non-smoker 14 h after death and was also stored at -85°C. Portions (20 g or 300 g) of frozen human liver were allowed to thaw at room temperature (20°C) and the resulting material was blended with 3 vols of ice-cold 50 mM-Tris/HCl buffer pH 7.5. A portion of thawed human lung (approx. 500 g) was blended in 2 vols of ice-cold 10 mM-sodium phosphate buffer pH 6.5. The cytosol and microsomal fractions were prepared by centrifugation using standard techniques.

Enzyme purification

(a) Human GST

The hepatic cytosolic GST were purified using, sequentially, DEAE-cellulose (at pH 8.0), S-hexylglutathione affinity chromatography (by the method of Mannervik & Guthenberg 1981), chromatofocusing and hydroxyapatite chromatography (Stockman et al., 1985). The pulmonary GST was isolated using, sequentially, SP-Sephadex (at pH 6.5), S-hexylglutathione affinity chromatography and chromatofocusing (pH 6.0 - 4.0).

(b) Rat GST

Rat GST were isolated to allow the preparation of antisera. The microsomal GST was purified by the method of Morgenstern et al., 1982. The preparation of the cytosolic forms has been described previously (Hayes, 1983, 1984, 1986).

Immunoblotting

This was performed using essentially the method devised by Towbin *et al*. (1979) and is described elsewhere (Hayes & Mantle, 1986a). The antisera used were obtained by standard techniques and their specificities have been described (Hayes & Mantle, 1986a).

RESULTS

Polymorphism of hepatic GST

Differences in the microsomal and cytosolic GST content of several livers were studied. Unlike the cytosolic GST, the microsomal form is not retained by S-hexylglutathione-Sepharose. Human microsomes were therefore studied directly, whereas the cytosolic GST were purified by affinity chromatography before examination.

The concentration of GST in microsomes prepared from five different human livers was examined by immunoblotting. Figure 1 shows a nitrocellulose blot of microsomes that had been subjected to SDS/PAGE. This demonstrated the presence of a 14 kDa polypeptide in all human specimens examined; this co-migrated during electrophoresis with the rat microsomal GST and possessed similar immunochemical properties.

Cytosolic GST represent a complex mixture of enzymes. The forms present in six different livers were examined by isoelectric focusing following S-hexylglutathione-Sepharose chromatography. Each purified GST pool produced a different electrophoretic pattern (Fig. 2); the position of purified marker GST is indicated. Proteins at the cathodal, or basic, end of the gel were observed in all samples. Protein that migrated with GST ε (B_1B_1, pI 8.9) was represented strongly in livers B, C, D and E, whereas livers A and F contained predominantly GST γ (B_2B_2, pI 8.4); all livers possessed GST δ (B_1B_2, pI 8.75). Although the 'basic' GST were present in all liver samples, 'neutral' and 'acidic' GST were only detected in certain livers; GST μ was found in livers A, B and D, GST ψ was only found in liver C and GST λ was very faintly observed in liver C.

Immunoblotting of cytosolic GST resolved by SDS/PAGE, showed that all livers contained GST subunits of 26.0 kDa that cross-reacted with anti-rat Ya-IgG. Livers A, B, C and D contained GST subunits of 26.7 kDa that cross-reacted with anti-rat Yb_1-IgG. Liver C showed very faint cross-reactivity with anti-rat Yf-IgG.

Purification of human GST

(a) Hepatic enzymes

For preparative purposes cytosol (normally from about 300 g of liver) was dialysed against 10 litres of 20 mM-Tris/HCl buffer pH 8.0 and applied to 4.4 cm x 80.0 cm columns of DEAE-cellulose that were equilibrated with the same buffer. The exchanger was developed with a 0 - 150 mM NaCl gradient. Figure 3 shows the profile obtained from liver D; the initial peak of activity, the 'flow through' fractions, contains both GST ε (B_1B_1) and GST δ (B_1B_2), the second peak of activity contains GST γ (B_2B_2) and the third peak of activity contains GST μ. GST Ψ was isolated from liver C and it, like GST μ, was eluted by the salt gradient in peak 3. Although GST ε (B_1B_1) and GST δ (B_1B_2) were both recovered from DEAE-cellulose in peak 1 they were resolved at a later stage in the purification scheme by chromatofocusing. Thus, the five major hepatic GST were each isolated using, sequentially DEAE-cellulose, S-hexylglutathione-Sepharose, chromatofocusing and hydroxyapatite. Table 1 summarises their chromatographic properties.

(b) Pulmonary GST

GST λ was isolated from 500 g of human lung. The cytosol, obtained following centrifugation at 100 000 g, was dialysed against 15 litres of 10 mM-sodium phosphate buffer pH 6.5 and applied to SP-Sephadex (4.4 cm x 80 cm) equilibrated with the same buffer. The activity that was not retained by this exchanger was purified by affinity chromatography and was finally eluted from the PBE 94 chromatofocusing gel at pH 4.8. The chromatographic properties of GST λ are included in Table 1.

Distinguishing features of cytosolic GST

SDS/PAGE of the purified human GST showed that they contain three subunit types; subunits of 24.8 kDa, 26.0 kDa and 26.7 kDa were observed (Fig. 4). All the enzymes were found to comprise two subunits of closely similar molecular mass. The 24.8 kDa polypeptide (GST λ) cross-reacted with antisera raised against the rat Yf GST subunit, the 26.0 kDa polypeptides (GST γ, δ and ε) cross-reacted with antisera raised against the rat Ya GST subunit and the 26.7 kDa polypeptides (GST μ and ψ) cross-reacted with antisera raised against rat Yb_1 subunits (Table 2).

All the GST purified catalyse the conjugation of 1-chloro-2, 4-dinitrobenzene with GSH but the subunit types can be identified catalytically using other substrates, such as ethacrynic acid, cumene hydroperoxide and p-nitrobenzyl chloride. Thus, the GST that comprises 24.8 kDa subunits (λ) has a high activity with ethacrynic acid as substrate. The GST that are composed of 26.0 kDa subunits (γ, δ and ε) possess glutathione peroxidase activity and are active with cumene hydroperoxide. The GST that contain 26.7 kDa subunits (μ and ψ) have a high activity towards p-nitrobenzyl chloride (Table 3).

DISCUSSION

Human liver expresses both microsomal GST and cytosolic GST. The microsomal enzyme appears to comprise subunits of 14.0 kDa that are immunochemically-related to the rat microsomal form described by Morgenstern et al. (1982, 1984). The cytosolic enzymes are composed of subunits of either 24.8, 26.0 or 26.7 kDa that are respectively immunochemically-related to the rat Yf, Ya and Yb GST subunits. The cytosolic GST are dimeric and all the forms identified were found to contain two subunits of equal size.

The cytosolic enzymes in man have been grouped into the 'basic', 'neutral' and 'acidic' GST but the pI ranges used to identify the three families are not clear cut. The observation that the 'basic' GST comprise 26.0 kDa (Ya) subunits, the 'neutral' GST comprise 26.7 kDa (Yb) subunits and the 'acidic' GST comprise 24.8 kDa (Yf) subunits suggests these enzymes might be more reliably classified, like the rat GST, according to their mobility during SDS/PAGE. A comparison between the equivalent rat and human GST revealed that the microsomal forms have a closely similar size as do the rat and human Yf subunits. However, differences in the electrophoretic mobilities of the rat Ya (25.5 kDa) and the human Ya (26.0 kDa) subunits as well as the rat Yb (26.3 kDa) and the human Yb (26.7 kDa) subunits are evident in Fig. 4.

Significant differences in the cytosolic GST content of the six livers examined were revealed by isoelectric focusing; variations were observed in both the 'basic' (pI 8.0 - 8.9) and the 'neutral' (pI 5.5 - 6.1) regions of the IEF gel.

Differences in the 'basic' GST expressed in individual livers have been shown to be due to the existence of two different Ya-type subunits, previously referred to as B_1 and B_2 (Stockman et al., 1985). During this study a marked difference was noted in the 'neutral' GST; the absence has been noted in certain livers of GST with either pI 6.1, or pI 5.5, or both. Subsequent purification of these enzymes showed the proteins of pI 6.1 and 5.5 both contained subunits of approximately 26.7 kDa, suggesting the existence of two Yb-type subunits in man.

Board (1981) used zymogram starch-gel analysis to investigate the variations in GST expressed in man and was the first to suggest the cytosolic forms could be ascribed to the existence of three loci; locus 1 is equivalent to the 'neutral' or Yb-containing forms, locus 2 is equivalent to the 'basic' or Ya-containing forms and locus 3 is equivalent to the 'acidic' or Yf-containing forms. The GST in human liver are primarily the products of locus 1 and 2. From his studies of the patterns of hepatic GST activity (towards 1-chloro-2,4-dinitrobenzene as electrophile) Board proposed that three autosomal alleles exist at locus 1 (one of which is null) and two autosomal alleles exist at locus 2. Our data are in broad agreement with that of Board but provide information about the polypeptide and catalytic differences between the GST in man.

The 'acidic' or Yf-containing GST are present in very low levels in normal human liver. This enzyme can be isolated from human lung; the Yf subunit is also present in rat lung (see Fig. 4). Similarly, in rat liver the Yf subunit is normally only present in very low levels. However, the Yf subunit, in both rat

and human livers, is specifically induced in experimentally-produced pre-neoplastic nodules and hepatomas (Faber, 1984; Satoh et al., 1985; Soma et al., 1985; Meyer et al., 1985; Jensson et al., 1985).

The genetic variation associated with the Ya- and Yb-containing GST may affect the susceptibility of different individuals to toxic insult by carcinogens and other environmental pollutants.

ACKNOWLEDGEMENTS

This work was funded by grants from the Medical Research Council (G8126392 SB) and the Scottish Home and Health Department (K/MRS/50/C570). We thank Mrs E Ward for typing this script.

REFERENCES

Awasthi, Y.C., Dao, D.D. & Saneto, R.P. (1980) Biochem. J. 191, 1-10.

Board, P.G. (1981) Am. J. Hum. Genet. 33, 36-43.

Chasseaud, L.F. (1979) Adv. Cancer Res. 29, 175-274.

Coles, B., Meyer, D.J., Ketterer, B., Stanton, C.A. & Garner, C. (1985) Carcinogenesis 6, 693-697.

Farber, E. (1984) Biochim. Biophys. Acta 738, 171-180.

Glatt, H., Friedberg, T., Grover, P.L., Sims, P. & Oesch, F. (1983) Cancer Res. 43, 5713-5717.

Habig, W.H. & Jakoby, W.B. (1981) Meth. Enzymol. 77, 398-405.

Hayes, J.D. (1983) Biochem. J. 213, 625-633.

Hayes, J.D. (1984) Biochem. J. 224, 839-852.

Hayes, J.D. (1986) Biochem. J. 233, 789-798.

Hayes, J.D. & Mantle, T.J. (1986a) Biochem. J. 233, 779-788.

Hayes, J.D. & Mantle, T.J. (1986b) Biochem. J. In the press.

Jakoby, W.B. (1978) Adv. Enzymol. Relat. Areas Mol. Biol. 46, 383-414.

Jensson, H., Eriksson, L.C. & Mannervik, B. (1985) FEBS Lett. 187, 115-120.

Jernstrom, B., Martinez, M., Meyer, D.J. & Ketterer, B. (1985) Carcinogenesis 6, 85-89.

Mannervik, B. (1985) Adv. Enzymol. 57, 357-417.

Mannervik, B. & Guthenberg, C. (1981) Meth. Enzymol. 77, 231-235.

Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M.K., Warholm, M. & Jornvall, H. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 7202-7206.

Meyer, D.J., Beale, D., Tan, K.H., Coles, B. & Ketterer, B. (1985) FEBS Lett. 184, 139-143.

Morgenstern, R., Guthenberg, C. & De Pierre, J.W. (1982) Eur. J. Biochem. 128, 243-248.

Morgenstern, R., Lundqvist, G., Andersson, G., Balk, L. & De Pierre, J.W. (1984) Biochem. Pharm. 33, 3609-3614.

Rannug, U., Sundvall, A. & Ramel, C. (1978) Chem.-Biol. Interactions 20, 1-16.

Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. & Sato, K. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 3964-3968.

Singh, S.V., Dao, D.D., Partridge, C.A., Theodore, C., Srivastava, S.K. & Awasthi, Y.C. (1985) Biochem. J. 232, 781-790.

Smith, G.J., Ohl, V.S. & Litwack, G. (1977) Cancer Res. 37, 8-14.

Soma, Y., Satoh, K. & Sato, K. (1986) Biochim. Biophys. Acta 869, 247-258.

Sparnins, V.L., Venegas, P.L. & Wattenberg, L.W. (1982) J. Natl. Cancer Inst. 68, 493-496.

Stockman, P.K., Beckett, G.J. & Hayes, J.D. (1985) Biochem. J. 227, 457-465.

Towbin, H., Staehlin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354.

Vander Jagt, D.L., Hunsaker, L.A., Garcia, K.B. & Roger, R.E. (1985) J. Biol. Chem. 260, 11603-11610.

Warholm, M., Guthenberg, C., Mannervik, B, Von Bahr, C. & Glaumann, H. (1980) Acta Chem. Scand. B34, 607-610.

Wolf, C.R., Berry, P.N., Nash, J.A., Green, T. & Lock, E.A. (1984) J. Pharmacol. Exp. Ther. 228, 202-208.

Table 1. Chromatographic properties of cytosolic GST isoenzymes

DEAE-cellulose and SP-Sephadex chromatography were both performed in 4.4 cm x 80.0 cm columns at pH 8.0 and pH 6.5 respectively. Chromatofocusing was carried out using 1.6 cm x 35.0 cm columns of PBE 94. Hydroxyapatite columns (1.6 cm x 19.0 cm) were developed with 10-250 mM sodium phosphate gradients at pH 6.7. Abbreviation; N.D. - not determined.

GST	Organ	Subunit size (kDa)	DEAE-cellulose	SP-Sephadex	Chromatofocusing (pH eluted)	Hydroxyapatite ([Na+] mmol/l)
Ψ	liver	26.7	peak 3	N.D.	7.1	85
μ	liver	26.7	peak 3	N.D.	7.8	100
γ	liver	26.0	peak 2	N.D.	N.D.	185
δ	liver	26.0	peak 1	N.D.	8.3	185
ε	liver	26.0	peak 1	N.D.	9.0	185
λ	lung	24.8	N.D.	Not retained	4.8	Not retained

Table 2. Immunochemical properties of GST

Antisera that were specific to the rat Ya (25.5 kDa), Yb_1 (26.3 kDa), Yc (27.5 kDa) and Yf (24.8 kDa) GST subunits were obtained in female New Zealand White rabbits and their crossreactivity with human GST was assessed by 'Western blotting' using a peroxidase labelled second antibody to visualise the immunoreactive subunits.

GST	Subunit size (kDa)	pI	Intensity of Cross-reactivity with antiserum against rat subunits			
			anti-Yb_1	anti-Ya	anti-Yf	anti-Yc
Ψ	26.7	5.5	+++	-	-	-
μ	26.7	6.1	+++	-	-	-
γ	26.0	8.4	-	+	-	-
δ	26.0	8.75	-	+	-	-
ε	26.0	8.9	-	+	-	-
λ	24.8	4.8	-	-	+++	-

Table 3. Discrimination between human GST using diagnostic substrates

GST	Subunit size (kDa)	pI	Relative activities with				
			CDNB	pNBC	tPBO	CH	EA
Ψ	26.7	5.5	+++++	+++	+++	-	±
μ	26.7	6.1	+++++	+++	+++	-	±
γ	26.0	8.4	++	-	-	+++	±
δ	26.0	8.75	++	-	-	++	±
ε	26.0	8.9	++	-	-	+	±
λ	24.8	4.8	++++	-	-	-	+++

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene
pNBC, p-nitrobenzyl chloride
tPBO, trans-4-phenyl-3-buten-2-one
CH, cumene hydroperoxide
EA, ethacrynic acid

Figure 1. Identification of human microsomal GST by 'Western blotting'.

Human microsomes that were obtained by centrifugation at 100 000 g from five separate livers were washed twice by repetitive ultracentrifugation. The pellets from the third centrifugation were re-suspended in 50 mM-Tris/HCl buffer pH 7.5 and subjected to SDS/PAGE. Tracks 1-5 contain 100 µg portions from livers A - E and track 6 contains 5 µg of purified rat microsomal GST. The polypeptides were transferred to nitrocellulose paper which was probed with antisera raised against the rat microsomal GST (subunit 14.0 kDa). The immunoreactive protein was visualised with a peroxidase-labelled second antibody and 4-chloro-1-naphthol as substrate.

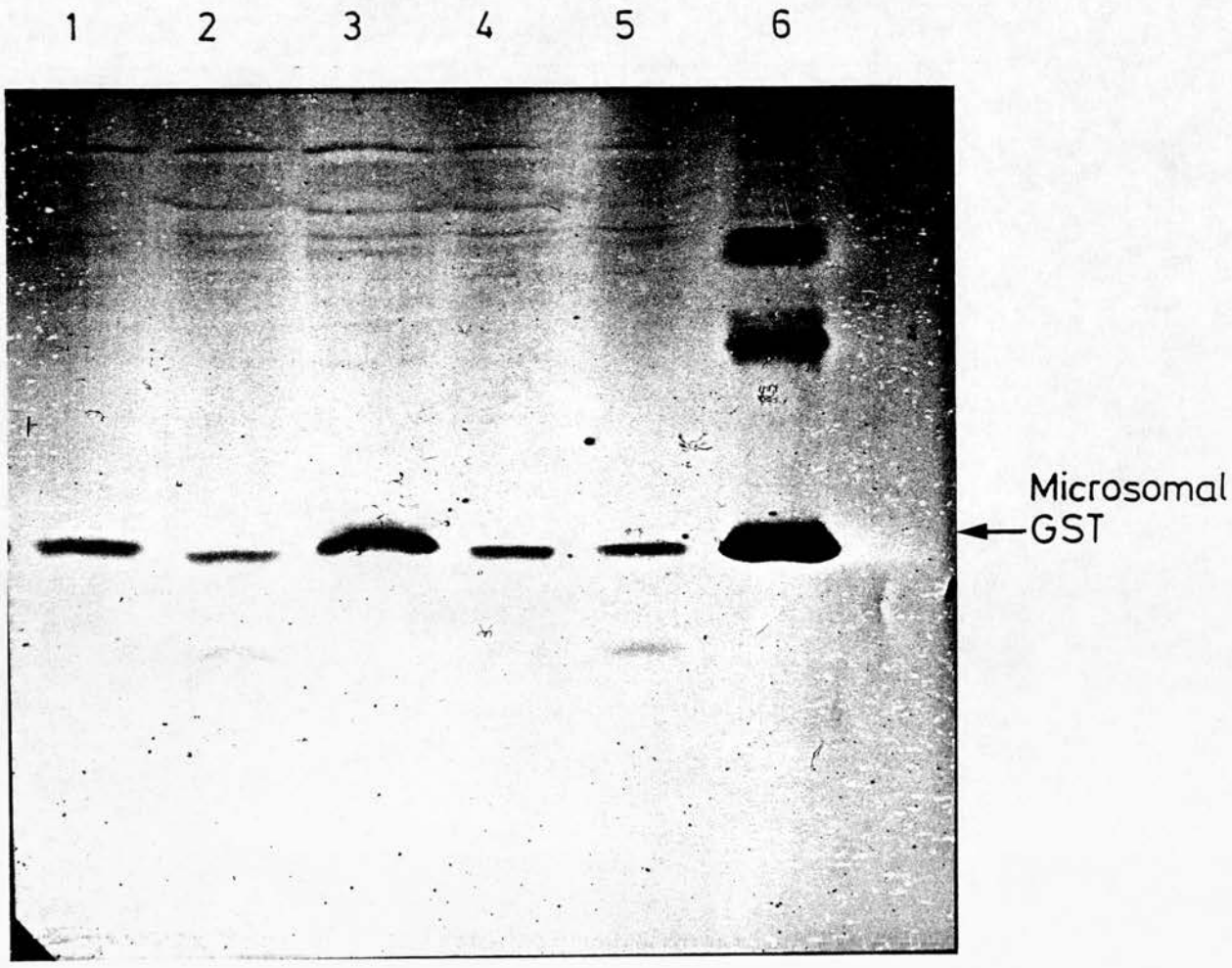


Figure 2. Isoelectric focusing of purified cytosolic GST

The cytosolic GST in six different liver specimens were separately purified by affinity chromatography on S-hexylglutathione-Sepharose that was developed by elution with 5 mM S-hexylglutathione. IEF was performed using a broad range gel (pH 3.5 - 9.5) in thin-layer 5% (w/v) polyacrylamide. The liver samples applied (A - F) are indicated. The tracks on the extreme left and right contain the pI calibration proteins.

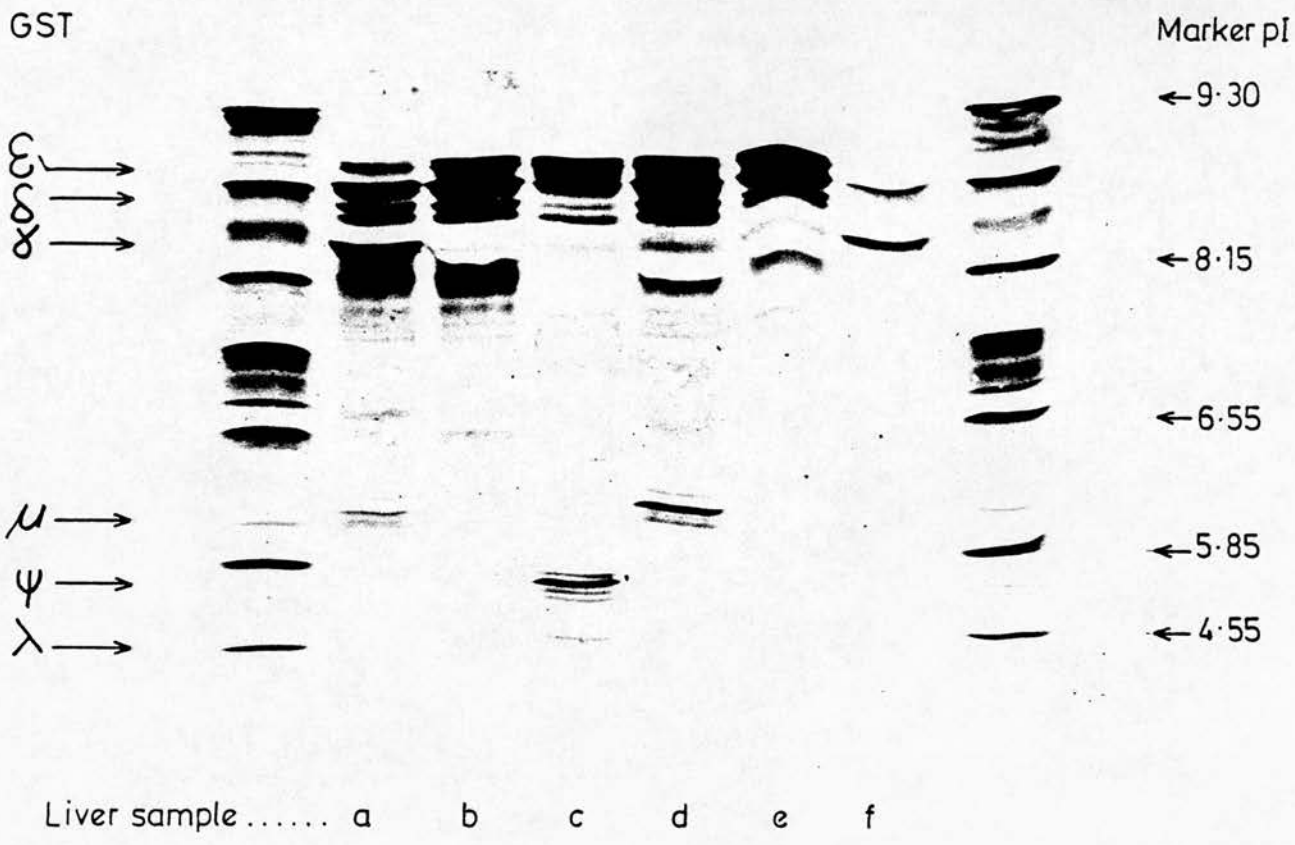


Figure 3. Resolution of cytosolic GST in human liver by DEAE-cellulose chromatography.

Hepatic cytosol from 300 g of tissue was dialysed against 10 litres of 20 mM-Tris/HCl buffer pH 8.0. The resulting material (about 500 ml) was applied to 4.4 cm x 80.0 cm columns of DEAE-cellulose. Fractions of 8.5 ml were collected and the activity with 1-chloro-2,4-dinitrobenzene (CDNB, ▲) determined. The Na^+ concentration (■) was also measured.

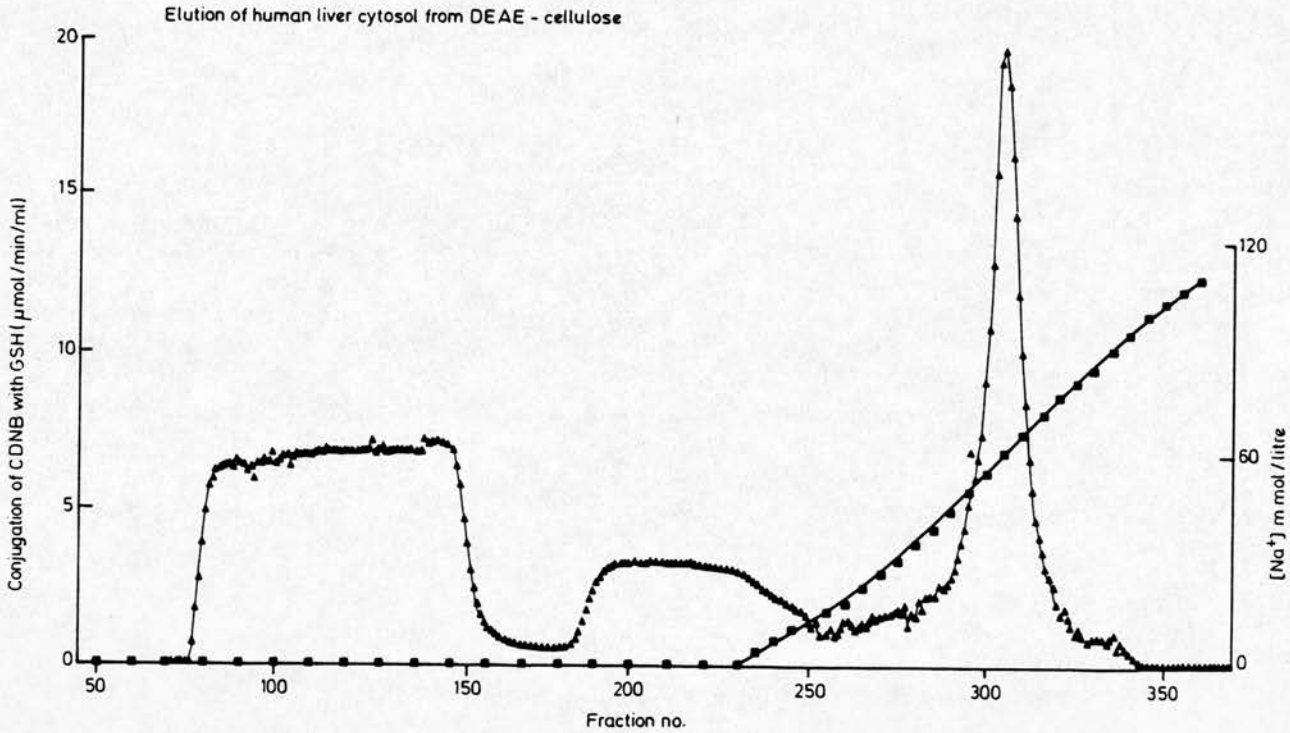
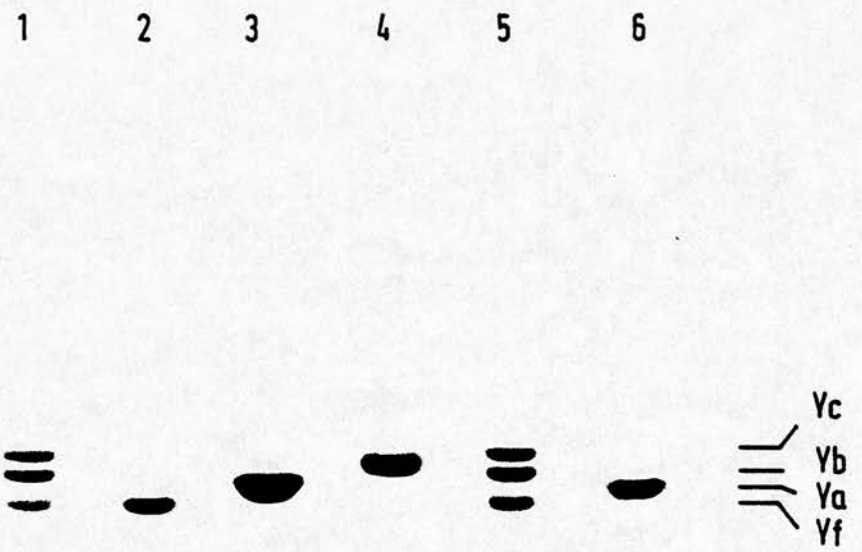


Figure 4. SDS/PAGE of cytosolic GST

SDS/polyacrylamide-gel electrophoresis was performed in 12% gels (w/v). Tracks 1 and 5 contain total rat lung GST. Tracks 2, 3 and 4 contain GST ('acidic'), GST μ ('neutral') and GST ε ('basic'). Track 6 contains rat liver GST L (YaYa protein). The position of the rat Yf (24.8 kDa), Ya (25.5 kDa), Yb (26.3 kDa) and Yc (27.5 kDa) is shown.



HUMAN GLUTATHIONE S-TRANSFERASES; A POLYMORPHIC GROUP OF DETOXIFICATION ENZYMES

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Detoxification by GST

The glutathione S-transferases (GST) are multi-functional proteins that protect against carcinogenesis (Smith et al., 1977). GST provide several detoxification mechanisms against cytotoxic drugs and carcinogens. Protection is afforded against these compounds by GST as a result of their catalytic properties and their ability to bind, both covalently and non-covalently, a wide variety of non-substrate ligands (Boyland & Chasseaud, 1969; Ketterer, 1982; Mannervik, 1985). Many toxic xenobiotics are strongly lipophilic and the GST-mediated conjugation of reduced glutathione (GSH) with such compounds facilitates their elimination from the body since the resulting metabolites are more water-soluble. GST binding of non-substrate lipophiles is also thought to serve a detoxification role either by preventing genotoxic compounds from interacting with DNA or by acting as carrier proteins assisting in the hepatic removal and biliary excretion of foreign compounds.

GST activity is represented by a large number of enzyme forms each of which is active towards a different spectrum of electrophilic compounds. Both cytosolic and microsomal GST exist and the different forms appear to serve distinct detoxification roles. In the rat, GST has been shown to catalyse the conjugation of GSH to a number of carcinogens and mutagens, including aflatoxin B_1-8,9-oxide and benzo(a)pyrene-7,

8-diol-9,10-epoxide, but marked differences in the specificities of the different isoenzymes have been reported (Glatt et al., 1983; Coles et al., 1985; Jernstrom et al., 1985). Differences also exist in the binding properties of the various GST for non-substrate ligands (Pattinson, 1981; Warholm et al., 1983; Sugiyama et al., 1985; Hayes & Mantle, 1986a). Only a limited number of GST bind, covalently, carcinogens such as metabolites of 4-dimethylaminoazobenzene or 3-methylcholanthrene; in the older literature, these forms have been called "ligandin" (Ketterer et al., 1967; Litwack et al., 1971; Carne et al., 1979; Hayes et al., 1979).

Genetic relationships between GST

Collectively, mammalian GST are encoded by at least four gene families; cytosolic GST appear to be the product of three families whereas microsomal GST is genetically distinct (Pickett et al., 1984; Ding et al., 1985; Mannervik et al., 1985; Morgenstern et al., 1985; Hayes & Mantle, 1986b). These enzymes have been most thoroughly investigated in the rat but those in man have also received considerable attention; recently, GST in the mouse have been studied by several groups of workers. Parallels can be drawn between the properties of GST from these three species; certain catalytic activities of GST that are members of the same gene family are conserved (Mannervik et al., 1985) as are some of their structural and immunochemical features (Hayes & Mantle, 1986c; Tu et al., 1986).

As a consequence of the large body of literature describing the rat GST, these enzymes have assumed the role of providing the "model" GST system. In this paper we shall use the nomenclature that was originally devised by Bass et al. (1977) to describe "ligandin"; this was later extended to describe the electrophoretic properties, during SDS/PAGE, of other purified GST subunits (Hayes et al., 1980; Scully & Mantle, 1980, 1981; Sheehan & Mantle, 1984). Cytosolic GST are dimeric and, in the rat, comprise six types of subunit, designated Ya (Mr 25 500), Yb (Mr 26 300), Yc (Mr 27 500), Yf (Mr 24 800), Yk (Mr 25 000) and Yn (Mr 26 000) (Hayes, 1984,

1986); the Yf subunit has also been called Yα (Tu et al., 1983) or Yp (Kitahara et al., 1984) and the Yn subunit has also been called Yβ (Li et al., 1986). The three classes of cytosolic GST can be grouped according to the subunits they comprise as follows: group I, Ya, Yc and Yk subunits; group II, Yb and Yn subunits; group III, Yf subunits. The group I enzymes may represent more than one gene family since not only is the expression of Ya and Yc polypeptides subject to distinct control mechanisms (Li et al., 1986) but also the Yk subunit has been shown to possess less sequence homology with Ya and Yc than these latter two subunits share with each other (Hayes, 1986). By contrast with the soluble enzymes, microsomal GST in the rat is composed of three identical subunits of Mr 17 000 (Morgenstern et al., 1982) and shares little significant sequence homology with the cytosolic subunits (Morgenstern et al., 1985). The activity of the microsomal GST is also distinct as its ability to catalyse the conjugation of GSH with 1-chloro-2,4-dinitro-benzene can be markedly increased by pretreatment of the enzyme with N-ethylmaleimide.

Human GST: identification of gene families

The different classes of GST in man can be resolved by isoelectric focusing and the most commonly used nomenclature for the human enzymes reflects this fact. The cytosolic GST have been divided into "basic", "neutral" or "near-neutral" and "acidic" enzymes (Kamisaka et al., 1975; Awasthi et al., 1980; Warholm et al., 1983; Mannervik 1985; Stockman et al., 1985; Soma et al., 1986); these classes of human GST are equivalent to the rat groups I, II and III, respectively. Electrophoresis of GST from human liver has shown that the "basic", "neutral" and "acidic" enzymes comprise subunits of Mr 26 000, 26 700 and 24 800, respectively. Like the rat Ya polypeptide, the subunits that comprise the human "basic" GST possess a cross-linker dependent migration during SDS/PAGE and display a relatively faster mobility in polyacrylamide-gels that contain low levels of NN'-methylene bisacrylamide (Hayes & Mantle, 1986c).

An earlier report suggesting that human

liver might lack microsomal GST (Morgenstern et al., 1984) does not appear to be correct. Immuno-blots of microsomal proteins from human liver have demonstrated the existence of a human polypeptide, with a size similar to the rat microsomal GST; this protein cross-reacts with antisera raised against the rat enzyme (L I McLellan & J D Hayes, unpublished results).

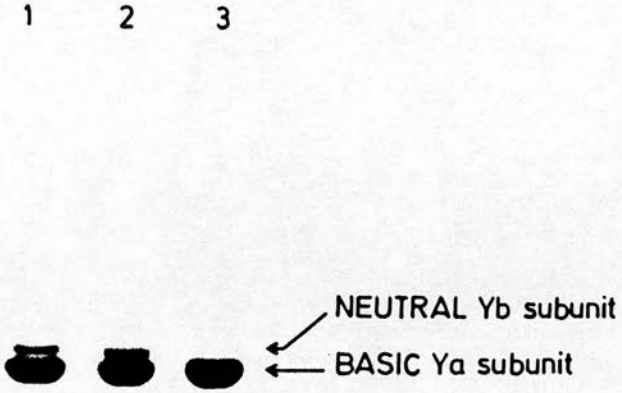
Human GST: polymorphism

The inter-individual differences in human GST have been studied by column isoelectric focusing (Warholm et al., 1980) and by zymogram starch-gel electrophoresis (Board, 1981; Strange et al., 1984). However, these analyses were performed on whole cytosol and provided no data about subunit differences that were responsible for the charge variations in the GST of different liver specimens. The use of affinity matrices such as S-hexylglutathione-Sepharose (Mannervik & Guthenberg, 1981) or glutathione-Sepharose (Simons & Vander Jagt, 1977; Vander Jagt et al., 1985) to isolate the cytosolic GST has facilitated investigations into the polymorphism associated with the soluble enzymes. GST are normally eluted from these columns as a single pool of activity. Further chromatographic procedures are needed to separate the individual isoenzymes.

Figure 1 shows SDS/PAGE of the total GST pool isolated by S-hexylglutathione-Sepharose affinity chromatography from three liver cytosols. All specimens were found to contain Ya-type subunits whereas only livers 1 and 2 expressed Yb-type subunits. Immunoblotting with anti-rat Ya-IgG showed cross-reactivity with all three livers whereas probing with anti-rat Yb-IgG only showed cross-reactivity with livers 1 and 2.

Isoelectric focusing in thin-layer slab polyacrylamide-gels (Figure 2) of the GST pools from the three livers demonstrated that all specimens contained multiple basic protein bands (pI values 8,4-8.9). Of the livers studied, liver 1 was unique as it possessed a strongly staining band of pI 6.1 (GST μ) and liver 2 was found to contain a characteristic protein band of

Figure 1. SDS/PAGE of the purified GST pools from three human liver cytosols.



pI 5.5 (GST Ψ). By contrast, liver 3 did not contain either of the pI 5.5 or 6.1 bands and isoelectric focusing showed the absence of any protein in this sample with pI <8.4. Liver 3, therefore, appears to represent a specimen that exhibits a null phenotype for the GST 1 locus. Board (1981) estimated that about 40% of individuals fail to synthesise any protein at the GST 1 ("neutral") locus.

The different classes of human GST elute from hydroxyapatite at characteristic positions.

Figure 2. Polyacrylamide-gel isoelectrc focusing of purified cytosolic GST pools from three human livers.

When the affinity-purified enzyme pools were examined by hydroxyapatite h.p.l.c., developed using a linear 10-350 mM-sodium phosphate gradient (pH 6.7), the GST from livers 1 and 2 yielded identical profiles. The enzymes from these livers resolved into 2 peaks that eluted at 37 min and 51 min (Figure 3); the peak that eluted at 37 min contained the "neutral" YbYb GST whereas the peak eluted at 51 min contained the "basic" YaYa GST. The enzyme from liver 3 eluted from hydroxyapatite as a single protein peak at 51 min. The hydroxyapatite h.p.l.c. profile obtained from a further liver, not represented in Figures 1-3, is shown since it contained trace amounts of the "acidic" YfYf GST that eluted at 15.5 min (Figure 4).

The "basic" and "neutral" cytosolic enzymes both partition into human microsomes; hence this subcellular fraction reflects the inter-individual differences that occur in the cytosol.

Figure 3. Resolution of "basic" and "neutral" GST from human liver by hydroxyapatite h.p.l.c.

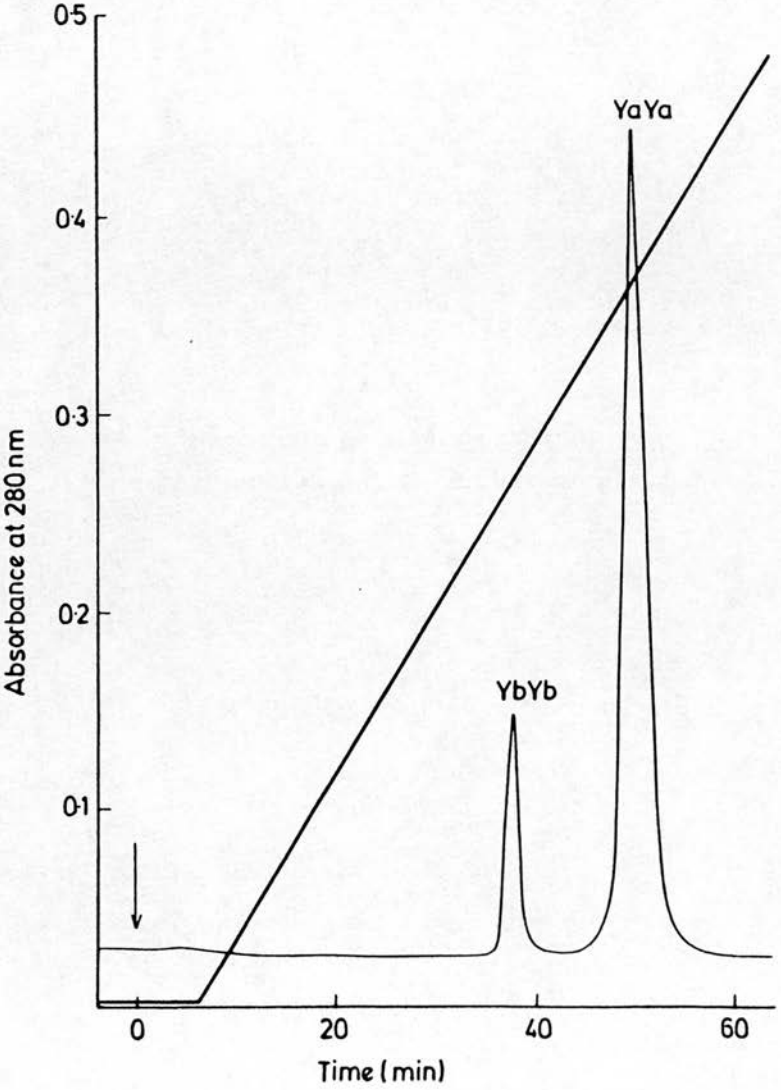
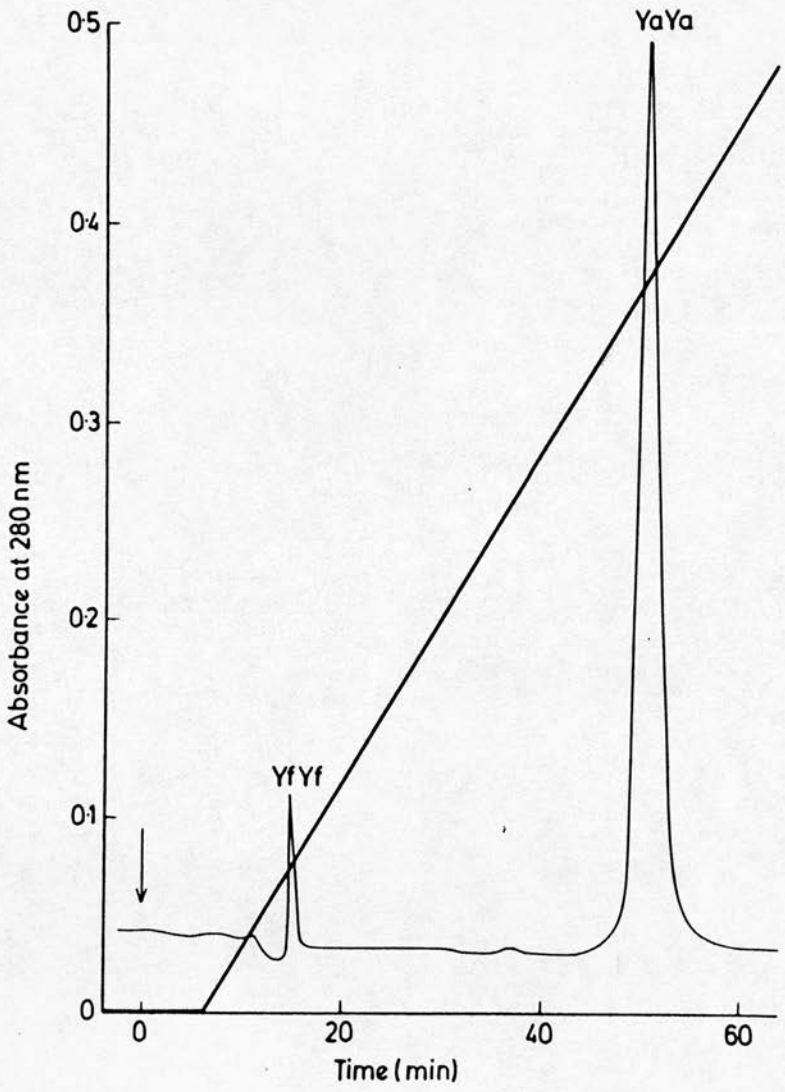


Figure 4. Resolution of "basic" and "acidic" GST from human liver by hydroxyapatite h.p.l.c.



owever, the polypeptide of Mr 17 000, equivalent o the rat microsomal GST described by orgenstern et al. (1985), has been found in all ivers studied. To date, we have examined the icrosomal GST content of six human livers and ave found no significant differences in the evels of the Mr 17 000 subunit.

uman GST: screening of hepatic GST content

The fact that variations exist between the ST found in human liver samples makes it esirable to employ a screening procedure to etermine which livers should be processed prior o undertaking a purification. A simple single-tep purification of the different GST classes an be achieved by employing a gradient elution o develop the S-hexylglutathione affinity atrix. Figure 5 shows the elution profile of iver 1 eluted from the affinity gel using a inear gradient of 0-0.25 mmol/l S-hexyl-lutathione between fractions 10-90 followed y a separate linear gradient of 0.25-5.0 mmol/l -hexylglutathione between fractions 91-120 see, Panel b). The "neutral" Yb-containing ST are retained by the affinity column signifi-antly longer than the "basic" Ya-containing nzymes; as these the "neutral" forms are clearly esolved, human livers that contain this class f GST can be simply identified.

Comparison between human and mouse (male, ALB/c strain) liver GST profiles reveals imilarities in the elution order of the ifferent enzyme classes from both species. The a-type subunits possess least affinity and the b-type subunits greatest affinity for the -hexyl-glutathione-Sepharose. Mouse Yf subunits xhibit intermediate affinity and elute between he Ya and the Yb-type subunits (Figure 5, Panel); similar results have been observed for the acidic" enzyme from human erythrocytes (GST ρ). he gradient elution of S-hexylglutathione-epharose allows glyoxalase to be resolved from ST ρ since it is adsorbed to the affinity matrix articularly tightly and is eluted, in fractions 4-99, by a much greater concentration of counter igand (2-3 mmol/l S-hexylglutathione) than GST. his differential elution of GSH-metabolising

Figure 5. Elution from S-hexylglutathione-Sepharose of GST from (a) mouse liver and (b) human liver.

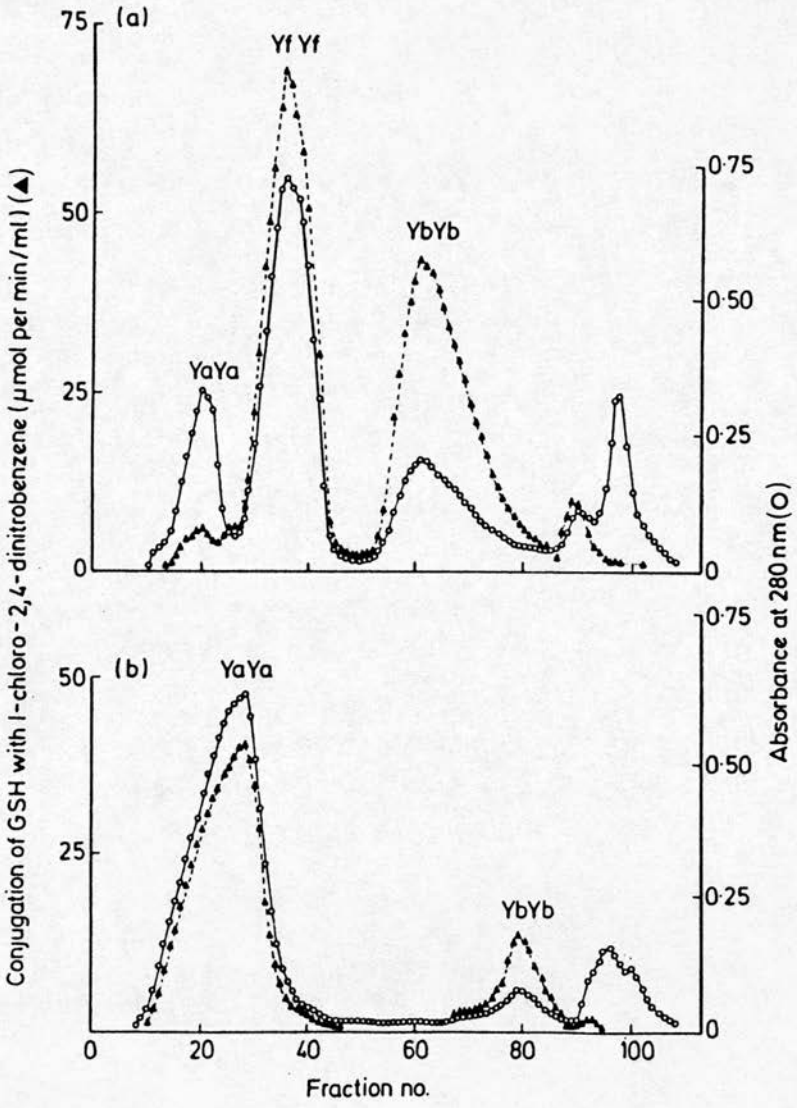
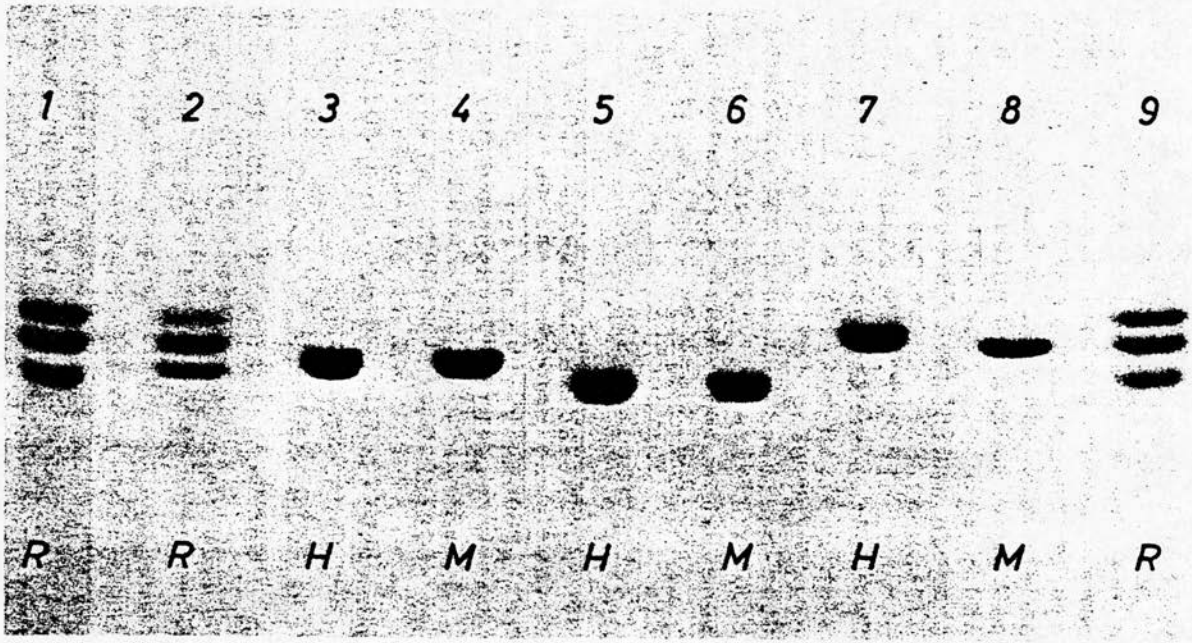


Figure 6. Comparison of electrophoretic properties of GST from rat (R), human (H) and mouse (M) tissues. The samples were analysed by SDS/PAGE as follows: 1, rat lung GST (Yf, Yb, Yc); 2, rat liver GST (Ya, Yb, Yc); 3, human "basic" GST (ε); 4, mouse GST YaYa; 5, human "acidic" GST (λ); 6, mouse GST YfYf; 7, human "neutral" GST (μ); 8, mouse GST YbYb; 9. rat lung GST (Yf, Yb, Yc).



proteins from S-hexylglutathione-Sepharose is a useful feature of the affinity gel when isolating GST since erythrocytes contain a particularly high level of glyoxalase.

Purification of GST and RIA

Large-scale isolation of "basic" Ya-containing and "neutral" Yb-containing GST is achieved from liver, whereas the most convenient source of "acidic" Yf-containing GST are lung or placenta (Dao et al., 1984; Stockman et al., 1985). The hepatic forms are purified using, sequentially, DEAE-cellulose (at pH 8.1), S-hexylglutathione-Sepharose and either CM-cellulose (at pH 6.7), for the "basic" GST, or chromatofocusing (pH 8.5-5.5) for "neutral" GST. By contrast, the "acidic" lung or placental forms are purified using SP-Sephadex (at pH 6.5), S-hexylglutathione-Sepharose and chromatofocusing (pH 5.5-4.0). SDS/PAGE analyses of the purified GST obtained by these methods are shown in Figure 6. This polyacrylamide-gel also demonstrates that mouse enzymes eluted from

Table 1. Immunochemical properties of human GST determined by RIA.

		Relative cross-reactivity of antiserum (%)			
GST*	pI	anti-ε (B_1B_1)	anti-γ (B_2B_2)	anti-μ	anti-λ
ε	8.9	100	0.2	<0.1	<0.1
δ	8.75	55	58	<0.1	<0.1
γ	8.4	0.3	100	<0.1	<0.1
μ	6.1	<0.1	<0.1	100	<0.1
ψ	5.5	<0.1	<0.1	100	<0.1
λ	4.8	<0.1	<0.1	<0.1	100

*GST ε, δ and γ have been designated B_1B_1, B_1B_2 and B_2B_2, respectively (Stockman et al., 1985).

S-hexylglutathione-Sepharose in fractions 27, 36 and 61 (see Figure 5a) possessed electrophoretic mobilities that were very similar to purified portions of human "basic" (GST ε, from liver), "acidic" (GST λ, from lung) and "neutral" (GST μ, from liver) enzymes, respectively. These similarities greatly facilitate subunit identification and suggest that certain structural features of GST are conserved between species. GST that belong to different classes are immunochemically distinct and little difficulty is normally experienced in discriminating between them. However, within a GST class, different subunits can combine to form heterodimers; in these circumstances problems are often found in identifying the various polypeptides. Radioimmunoassay has proved valuable in discriminating between the two "basic" Ya-type subunits, defined as B_1 and B_2 (Table 1). These two subunits can combine to form B_1B_1, B_1B_2 and B_2B_2; it has been proposed (Stockman *et al.*, 1985) that these forms correspond to the GST ε, δ and γ, respectively, that were first described by Kamisaka *et al.* (1975).

CONCLUSIONS

GST are a complex group of enzymes that, in man, are encoded by at least 4 gene families. It is highly likely that the marked polymorphism associated with the human enzymes will have both pathological and pharmacological implications. However, considerably more work is required before the molecular basis of the inter-individual variations in human GST expression are fully understood.

ACKNOWLEDGEMENTS

This work was funded by grants from the Scottish Home and Health Department (K/MRS/50/C570) and the Medical Research Council (G8126392 SB).

REFERENCES

Awasthi, Y.C., Dao, D.D. & Saneto, R.P., 1980, Biochemical Journal, 191, 1.
Bass, N.M., Kirsch, R.E., Tuff, S.A., Marks, I. & Saunders, S.J., 1977, Biochimica Biophysica Acta, 492, 163.
Board, P.G., 1981, American Journal Human Genetics, 33, 36.
Boyland, E. & Chasseaud, L.F., 1969, Advances Enzymology Related Areas Molecular Biology, 32, 173.
Carne, T., Tipping, E. & Ketterer, B., 1979, Biochemical Journal, 177, 433.
Coles, B., Meyer, D.J., Ketterer, B., Stanton, C.A. & Garner, C., 1985, Carcinogenesis, 6, 693.
Dao, D.D., Partridge, C.A., Kurosky, A. & Awasthi, Y.C., 1984, Biochemical Journal, 221, 33.
Ding, G.J.-F., Lu, A.Y.H. & Pickett, C.B., 1985, Journal Biological Chemistry, 260, 13268.
Glatt, H., Friedberg, T., Grover, P.L., Sims, P. & Oesch, F.,1983, Cancer Research, 43, 5713.
Hayes, J.D., 1984, Biochemical Journal, 224, 839.
Hayes, J.D., 1986, Biochemical Journal, 233, 789.
Hayes, J.D. & Mantle, T.J., 1986a, Biochemical Journal, 233, 407.
Hayes, J.D. & Mantle, T.J., 1986b, Biochemical Journal, 233, 779.
Hayes, J.D. & Mantle, T.J., 1986c, Biochemical Journal, 237, 731.
Hayes, J.D., Strange, R.C. & Percy-Robb, I.W., 1979, Biochemical Journal, 181, 699.
Hayes, J.D., Strange, R.C. & Percy-Robb, I.W., 1980, Biochemical Journal, 185, 83.
Jernstrom, B., Martinez, M., Meyer, D.J. & Ketterer, B., 1985, Carcinogenesis, 6, 85.
Kamisaka, K., Habig, W.H., Ketley, J.N. & Arias, I.M. & Jakoby, W.B., 1975, European Journal Biochemistry, 60, 153.
Ketterer, B., 1982, Drug Metabolism Reviews, 13, 161.
Ketterer, B., Ross-Mansell, P. & Whitehead, J.K., 1967, Biochemical Journal, 103, 316.
Kitahara, A., Satoh, K., Nishimura, K., Ishikawa, T., Ruike, K., Sato, K., Tsuda, H. & Ito, N., 1984, Cancer Research, 44, 2698.

Li, N.-q., Reddana, P., Kedam, T., Reddy, C.C. & Tu, C.-P.D., 1986, Journal Biological Chemistry, 261, 7596.
Litwack, G., Ketterer, B. & Arias, I.M., 1971, Nature (London), 234, 466.
Mannervik, B., 1985, Advances Enzymology Related Areas Molecular Biology, 57, 357.
Mannervik, B. & Guthenberg, C., 1981, Methods Enzymology, 77, 231.
Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M.K., Warholm, M. & Jornvall, H., 1985, Proceedings National Academy Science, 82, 7202.
Morgenstern, R., Guthenberg, C. & De Pierre, J.W., 1982, European Journal Biochemistry, 128, 243.
Morgenstern, R., Lundqvist, G., Andersson, G., Balk, L. & De Pierre, J.W., 1984, Biochemical Pharmacology, 33, 3609.
Morgenstern, R., De Pierre, J.W. & Jornvall, H., 1985, Journal Biological Chemistry, 260, 13976.
Pattinson, N.R., 1981, Biochemical Biophysical Research Communications, 102, 403.
Pickett, C.B., Telakowski-Hopkins, C., Ding, G., Argenbright, L. & Lu, A.Y.H., 1984, Journal Biological Chemistry, 259, 5182.
Scully, N.C. & Mantle, T.J., 1980, Biochemical Society Transactions, 8, 451.
Scully, N.C. & Mantle, T.J., 1981, Biochemical Journal, 193, 367.
Sheehan, D. & Mantle, T.J., 1984, Biochemical Journal, 218, 893.
Simons, P.C. & Vander Jagt, D.L., 1977, Analytical Biochemistry, 82, 344.
Smith, G.J., Ohl, V.S. & Litwack, G., 1977, Cancer Research, 37, 8.
Soma, Y., Satoh, K. & Sato, K., 1986, Biochimica Biophysica Acta, 869, 247.
Stockman, P.K., Beckett, G.J. & Hayes, J.D., 1985, Biochemical Journal, 227, 457.
Strange, R.C., Faulder, C.G., Davis, B.A., Hume, R., Brown, J.A.H., Cotton, W. & Hopkinson, D.A., 1984, Annals Human Genetics, 48, 11.

Sugiyama, Y., Sugimoto, M., Stolz, A. & Kaplowitz, N., 1984, Biochemical Pharmacology, 33, 3511.

Tu, C.-P.D., Matsushima, A., Li, N.-q., Rhoads, D.M., Srikumar, K., Reddy, A.P. & Reddy, C.C., 1986, Journal Biological Chemistry, 261, 9540.

Vander Jagt, D.L., Hunsaker, L.A., Garcia, K.B. & Royer, R.E., 1985, Journal Biological Chemistry, 260, 11603.

Warholm, M., Guthenberg, C., Mannervik, B. von Bahr, C. & Glaumann, H., 1980, Acta Chemica Scandinavica, B34, 607.

Warholm, M., Guthenberg, C. & Mannervik, B., 1983, Biochemistry, 22, 3610.

Biochimica et Biophysica Acta 874 (1986) 1–12
Elsevier

BBA 32545

Variations in the glutathione *S*-transferase subunits expressed in human livers

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(Received 9 June 1986)

Key words: Glutathione *S*-transferase; Drug-metabolising enzyme; Enzyme polymorphism; Immuno-blotting; (Human liver)

Human livers express a variety of cytosolic glutathione *S*-transferase isoenzymes. The enzymes are subject to a marked polymorphism and the polypeptide basis of the differences in glutathione *S*-transferase content of individual livers has been investigated by Western blotting, hydroxyapatite HPLC and isoelectric focusing. Collectively, the livers examined contained three distinct groups of cytosolic glutathione *S*-transferase. The three classes of enzyme contain subunits of different molecular mass; subunits of 24.8 kDa (Yf), 26.0 kDa (Ya) and 26.7 kDa (Yb) were found to belong to the 'acidic-type', 'basic-type' and 'neutral-type' glutathione *S*-transferase, respectively. All livers studied contained 26.0 kDa subunits (Ya or 'basic') but significant differences in the isoelectric points of this group of proteins were demonstrated. Five of the eight livers examined expressed 26.7 kDa subunits (Yb or 'neutral'); the native enzymes had p*I* values of either 6.1 or 5.5, and were isolated by hydroxyapatite HPLC. Two of the livers possessed 24.8 kDa subunits (Yf or 'acidic'), and the native enzyme, which had a p*I* of 4.8, was also purified by hydroxyapatite HPLC. Before undertaking a glutathione *S*-transferase purification it is advisable to determine the GST isoenzyme content of a number of livers. The suitability of the methods described in the present study for use as screening procedures is discussed.

Introduction

Glutathione *S*-transferases (EC 2.5.1.18) are a group of enzymes that detoxify a wide range of potentially harmful electrophiles by conjugation with glutathione [1,2]. This reaction is the first step in the formation of mercapturic acids, a pathway that is important as a means of rendering hydrophobic xenobiotics water-soluble and thereby aiding their elimination from the body [3,4].

The glutathione *S*-transferase enzymes are present in many species; however, they have been most extensively studied in the rat. At least 12 cytosolic glutathione *S*-transferase isoenzymes have been described in rat liver [5,6]; each of these comprise two subunits and both homo- and heterodimers exist [7–10]. The enzymes in rat cytosol are coded for by three multi-gene families and only subunits of the same gene family can hybridize [5,9,11].

In man the cytosolic enzymes can be divided, on the basis of their isoelectric points, into three groups, the 'basic', the 'neutral' and the 'acidic' [2,12]. The relationship between the forms that comprise these three groups is not clear. Contrasting reports have appeared in the literature about many of the structural features of these enzymes; confusion exists about the immunochemical properties of human glutathione *S*-transferase, about the subunit composition of individual glutathione

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S-transferases and about the polymorphism associated with the hepatic enzymes in man. For instance, 'acidic' and 'basic' glutathione *S*-transferases have been conflictingly reported as either immunochemically distinct [2,13] or, alternatively, immunochemically inter-related enzymes [14]. Furthermore, it has been proposed that all the 'basic' glutathione *S*-transferases are heterodimers [15,16], composed in certain cases of a 'basic' and an 'acidic' subunit, yet others have identified two distinct 'basic' homodimers, which are structurally distinct from the 'acidic' subunits [17,18]. Differences in the glutathione *S*-transferase content of individual livers have been reported, particularly the neutral enzyme, μ, [19,20]. However, in two separate studies, Awasthi and co-workers found no significant differences in the glutathione *S*-transferase content of six livers [14,15].

In the present study we have developed several rapid screening procedures that allow the glutathione *S*-transferase isoenzymes expressed in different human livers to be identified. The cytosolic isoenzymes present in each of eight human liver samples were investigated with respect to their electrophoretic, immunochemical and chromatographic properties.

Materials and Methods

All chemicals used were readily available commercially.

Human liver samples were from individuals who died either from accidental causes or from a disease that did not involve the liver. The ages and sex of these individuals are shown in Table I. The livers were obtained within 2 h of death and, following snap freezing in liquid nitrogen, were stored at -80°C until required.

Isolation of glutathione S-transferase. Portions (10–50 g) of individual human livers were allowed to thaw at 20°C, and 20% homogenates (w/v) were separately prepared in ice-cold 20 mM Tris-HCl buffer, pH 7.0. The extracts were centrifuged for 20 min at $10\,000 \times g$ (4°C) and the pellets discarded. The resulting supernatants were re-centrifuged for 60 min at $100\,000 \times g$ (4°C); the post-$100\,000 \times g$ supernatants were collected and are referred to as cytosol.

The various cytosols were dialysed for 24 h at 4°C, against two changes (each of 4 litres) of 20 mM Tris-HCl buffer, pH 7.8, which contained 200 mM NaCl (buffer A). The dialysed cytosols were subjected to affinity chromatography on columns (1.6×15.0 cm) of *S*-hexylglutathione-Sepharose as described elsewhere [11,21]. The glutathione *S*-transferase enzymes bound to each column were washed with about 500 ml of buffer A before being eluted with a solution of 5 mM *S*-hexylglutathione in the same buffer. The fractions eluted that contained purified glutathione *S*-transferase were combined and the catalytic activities, immunochemical properties, electrophoretic mobilities, and chromatographic behaviour on hydroxyapatite HPLC, of the isoenzymes in different livers were examined (see below).

General analytical. Glutathione *S*-transferase activity was measured at 37°C using either 1-chloro-2,4-dinitrobenzene, *trans*-4-phenyl-3-buten-2-one or ethacrynic acid as substrate [22]. Protein concentration was measured by the method of Bradford [23].

SDS-polyacrylamide-gel electrophoresis was performed by the method of Laemmli [24]. Normally the resolving gel comprised 12% polyacrylamide (w/v) and contained 0.32% (w/v) *N*,*N*′-methylenebisacrylamide (C_{Bis} 2.6%) but, on occasions, a resolving gel that comprised 15% polyacrylamide (w/v) and contained 0.09% (w/v) *N*,*N*′-methylenebisacrylamide (C_{Bis} 0.6%) was employed, since the use of low levels of cross-linker has been shown to improve the resolution between the 'basic' and the 'neutral' subunits [25].

Immuno-blotting. This was performed by the method of Towbin et al. [26]. The antisera against the cytosolic glutathione *S*-transferase were raised, by standard immunochemical protocols, against homogeneous isoenzymes. The purified enzymes used for this purpose were as follows: ε (B_1B_1, a 'basic' enzyme), γ (B_2B_2, a 'basic' enzyme), μ (a 'neutral' enzyme) and λ (an 'acidic' enzyme); these were isolated by the methods described in Refs. 11, 17 and 27. Each antiserum only cross-reacted with its own subunit type.

The cytosolic rat liver and rat lung glutathione *S*-transferase pools that were isolated by *S*-hexylglutathione-Sepharose chromatography were used as positive controls that permitted calculation of the molecular mass of cross-reacting

TABLE I

DATA ON HUMAN LIVER SAMPLES

Patient No.	Initials	Age	Sex	Smoker	Drugs	Cause of death
1	JQ	40	female	no	ampicillin, sulphonamide, alcuronium, phenoperidine, dexamethasone	road accident
2	AD	68	female	no	flucooxocillin, phenytoin, alcuronium, phenopseridine	road accident
3	CN	36	male	no	cefotaxime, phenoperidine, alcuronium	oligodendrogliona
4	HH	55	female	no	insulin, dexamethasone, phenoperidine, frusemide	
5	AC	42	male	no	dexamethasone, phenytoin	road accident
6	RA	34	male	no	dexamethasone	road accident
7	JH	49	male	yes, 20 daily	none	subarachnoid haemorrhage
8		34	male	yes: no. not known	dopamine, ampicillin, mannitol, ampicillin	road accident

species. The major rat liver glutathione *S*-transferase isolated by this affinity matrix comprises Ya (25.5 kDa), Yb (26.3 kDa) and Yc (27.5 kDa) subunits, whereas the major rat lung glutathione *S*-transferase comprises Yf (24.8 kDa), Yb (26.3 kDa) and Yc (27.5 kDa) subunits [11,25].

Radioimmunoassay (RIA). RIA of B_1 and B_2 subunits [28] was performed on fractions purified by *S*-hexylglutathione affinity chromatography.

Isoelectric focusing. This was carried out using a broad-range gel (pH 3.5–9.5) in thin-layer 5% (w/v) polyacrylamide. The electrophoretic apparatus used was from LKB Produkter, Bromma, Sweden. The protein p*I* calibration standards were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and included trypsinogen (p*I* 9.30), lentil lectin (p*I* 8.65, 8.45, 8.15), horse myoglobin (p*I* 7.35, 6.85), human carbonic anhydrase B (p*I* 6.55), bovine carbonic anhydrase B (p*I* 5.85), β-lactoglobulin A (p*I* 5.20), soybean trypsin inhibitor (p*I* 4.55) and amyloglucosidase (p*I* 3.50). Purified human glutathione *S*-transferase was run in parallel to aid enzyme identification.

Hydroxyapatite HPLC. The different glutathione *S*-transferase types, purified by *S*-hexylglutathione affinity chromatography, were resolved by chromatography on Bio-Gel HPHT (Bio-Rad Laboratories). The column was equilibrated with 10 mM sodium phosphate, pH 6.7, and developed using a 10–350 mM sodium phosphate gradient; the HPLC system employed was from Waters Associates (Instruments), Northwich, Cheshire, U.K. Fractions of 0.5 ml were collected and the flow rate was 0.5 ml/min. Details of the method employed are described elsewhere [29].

Results

Purification of cytosolic glutathione S-transferase

The *S*-hexylglutathione-Sepharose 6B affinity matrix retained more than 85% of the cytosolic glutathione *S*-transferase activity from each liver, as assessed using 1-chloro-2,4-dinitrobenzene and GSH as substrates. Table II shows the activities of the different affinity-purified glutathione *S*-transferase pools for three substrates. SDS-PAGE of these purified enzymes from different livers (Fig. 1) shows that all eight samples contain a major polypeptide (26.0 kDa) with an electrophoretic mobility similar to that of the rat Ya glutathione *S*-transferase subunit. However, marked differences exist between the individual livers in their content of subunits with slower mobility than the 26.0 kDa polypeptide. For example, livers 1, 2, 3, 4 and 6 (but not 5, 7 or 8) contained a polypeptide of 26.7 kDa that migrated between the rat Yb and Yc subunits. Small amounts of a polypeptide with molecular mass 29.0–30.0 kDa are also observed in livers 3, 4 and 7, but purification of these monomers by hydroxyapatite HPLC (see below) suggests that these may not represent glutathione *S*-transferase subunits. The presence of the small 24.8 kDa glutathione *S*-transferase subunit, referred to as Yf [5,6,25], was not detected in any liver sample using SDS-PAGE as a screening method.

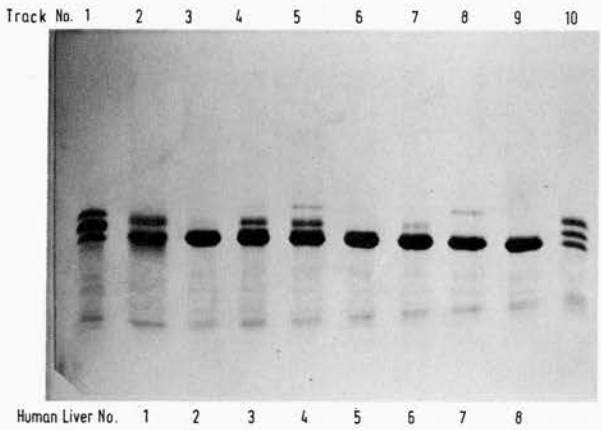

Fig. 1. Electrophoretic analysis of glutathione *S*-transferase purified by affinity chromatography. SDS-polyacrylamide-gel electrophoresis was performed by the method of Laemmli [24]. The resolving gel comprised 12% polyacrylamide (w/v). The rat liver subunit markers Ya (25.5 kDa), Yb (26.3 kDa) and Yc (27.5 kDa) were loaded in tracks 1 and 10. The glutathione *S*-transferase purified by *S*-hexylglutathione affinity chromatography (8 μg) from livers 1–8 were loaded in tracks 2–9 respectively.

Immunochemical identification of cytosolic glutathione S-transferase

Immuno-blots of the *S*-hexylglutathione-affinity-purified cytosolic enzymes are shown in Fig. 2. Antisera against the basic enzyme glutathione *S*-transferase ε (B_1B_1) cross-reacted with polypeptides of molecular mass approximately 26.0 kDa in the preparations from all eight livers. A similar pattern of cross-reactivity was observed when the liver cytosols were probed with antisera raised against glutathione *S*-transferase γ (B_2B_2).

Antisera against the 'neutral' enzyme, glutathione *S*-transferase μ, cross-reacted with livers 1, 2, 3, 4 and 6 but not with livers 5, 7 or 8 (Fig. 2b); the cross-reacting species possessed a molecular

TABLE II

GLUTATHIONE *S*-TRANSFERASE ACTIVITY IN FRACTIONS PURIFIED BY AFFINITY CHROMATOGRAPHY

The enzyme assays were performed as described in Ref. 22. A value of zero denotes a result where the rate of thioether formation was not discernibly different from the blank rate when a minimum of 80 μg protein was used. n.d., not determined, due to paucity of material.

Substrate	Specific activity (μmol/min per mg protein)							
	Human liver							
	1	2	3	4	5	6	7	8
1-Chloro-2,4-dinitrobenzene	21.7	25.5	38.1	35.3	23.6	17.9	42.6	65.1
trans-4-Phenyl-3-buten-2-one	n.d.	0.0013	0.0022	0.0013	0	0.0014	0	0
Ethacrynic acid	n.d.	0.030	0.040	0.040	0.023	0.014	0.110	0.086

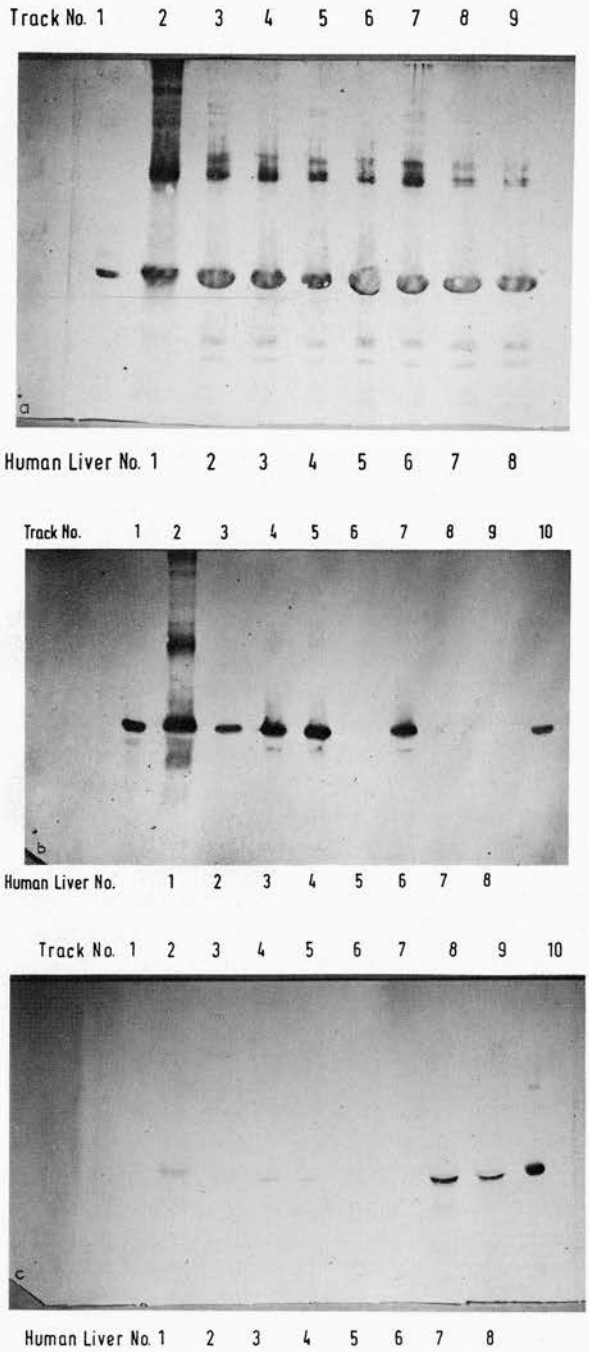

Fig. 2. Immunochemical identification of cytosolic glutathione *S*-transferase purified by affinity chromatography. Immunoblots of the *S*-hexylglutathione-purified cytosolic enzymes were performed by the method of Towbin et al. [26]. The electrophoresis was carried out in 12.0% (w/v) polyacrylamide gel. Rat liver and rat lung pools isolated by *S*-hexylglutathione chromatography were loaded in tracks 1 and 10 respectively, with the exception of (a) in which the rat lung pool was absent. Isoenzymes (8 μg) from human livers 1–8 were loaded in tracks 2–9 respectively. The glutathione *S*-transferases were then probed with antisera to (a) ε, (b) μ and (c) λ.

mass of about 26.7 kDa. Antisera against the 'acidic' enzyme, λ, showed an intense staining with the glutathione *S*-transferase from livers 7 and 8, whereas livers 1, 3 and 4 exhibited only a trace cross-reactivity and livers 2, 5 and 6 possessed no cross-reactivity (Fig. 2c). The polypeptides that cross-reacted with anti-glutathione *S*-transferase λ migrated with the Yf subunit 24.8 kDa in rat lung that was run in parallel (Fig. 2c, track 10; the rat lung subunit markers used were Yf, Yb, Yc). No cross-reactivity with anti-glutathione *S*-transferase λ was observed with rat liver glutathione *S*-transferase (Fig. 2c, track 1; rat liver enzyme comprises Ya, Yb, Yc subunits).

The affinity-purified fractions were also analysed by RIA. The ratios of the amounts of B_2 to B_1 subunits are shown in Table III. From these data it is apparent that material purified from liver 6 contains a proportionately larger amount of B_2 subunit than is present in the other affinity-purified fractions.

Identification of cytosolic glutathione S-transferase by hydroxyapatite HPLC

Bio-Gel HPHT resolved the isoenzymes in the various pools into three peaks (Fig. 3). Peak 1 eluted at 15.5 min, peak 2 at 37.0 min and peak 3 at 51.0 min. Dot-blotting portions of these peaks with anti-ε (basic), anti-μ (neutral) and anti-λ (acidic) showed that peaks, 1, 2 and 3 contained 'acidic', 'neutral' and 'basic' glutathione *S*-transferase, respectively.

Several examples of the profiles obtained from the Bio-Gel HPHT column are shown in Fig. 3. These demonstrate that the relative amounts of different peaks vary considerably between individual liver specimens. For example, in liver 1 the 'neutral' (Peak 2) represents the major isoenzyme(s), whereas in liver 2 the 'neutral' is quantitatively insignificant and the 'basic' represents about 95% of the total enzyme present. Likewise, the 'acidic' accounts for about 3–5% of the enzyme in livers 7 and 8 but is not detected in livers

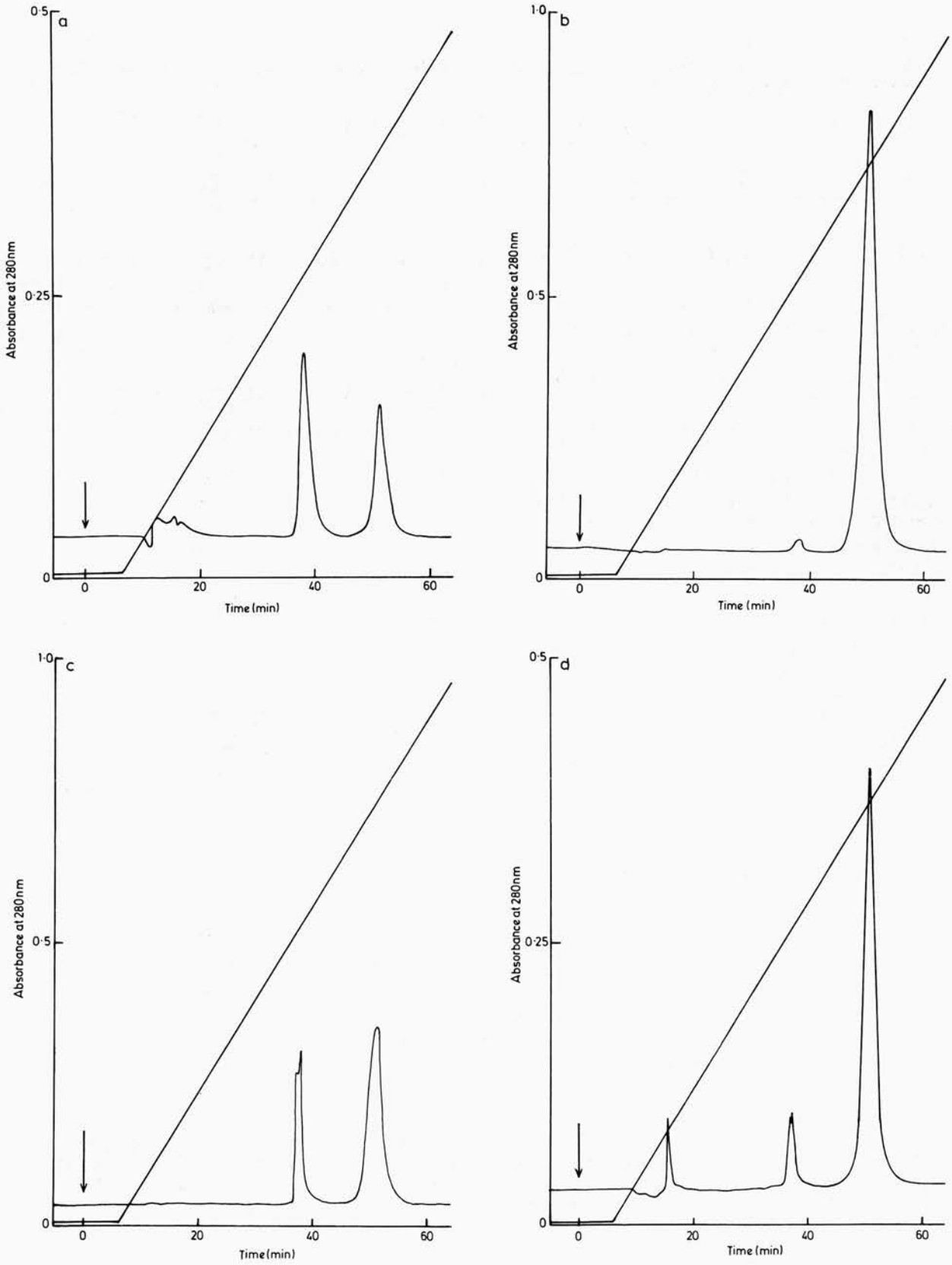
a
0·5
0·25
0
Absorbance at 280nm
0
20
40
60
Time (min)
b
1·0
0·5
0
Absorbance at 280nm
0
20
40
60
Time (min)
c
1·0
0·5
0
Absorbance at 280nm
0
20
40
60
Time (min)
d
0·5
0·25
0
Absorbance at 280nm
0
20
40
60
Time (min)

TABLE III

RADIOIMMUNOASSAY OF 'BASIC' GLUTATHIONE *S*-TRANSFERASE SUBUNITS PRESENT IN FRACTIONS PURIFIED BY AFFINITY CHROMATOGRAPHY

	Human liver : *S*-hexylglutathione affinity-purified fractions							
	1	2	3	4	5	6	7	8
Ratio of B_2/B_1 subunits	0.28	0.54	0.53	0.16	0.90	1.43	0.43	0.51

2, 3 or 4. A summary of the findings is shown in Table IV.

The 1-chloro-2,4-dinitrobenzene : GSH conjugating activities of peaks 1, 2 and 3 obtained from the different livers were examined and shown to express glutathione *S*-transferase activity, with the exception of a protein which eluted in the 'peak 2 region' of the HPHT chromatogram obtained from liver 7.

SDS-PAGE (Fig. 4) demonstrated that the peak 1 enzymes, the 'acidic' glutathione *S*-transferase, comprise Yf subunits (24.8 kDa). The peak 2 enzymes, the 'neutral' glutathione *S*-transferase, comprise Yb subunits (26.7 kDa) (the peak 2-like material from liver 7 also contained a polypeptide of molecular mass 29.0 kDa, but this did not express glutathione *S*-transferase activity towards CDNB). The peak 3 enzymes, the 'basic' glutathione *S*-transferase, are composed of subunits of molecular mass 26.0 kDa. Certain physical and immunochemical properties of the enzymes resolved by hydroxyapatite HPLC are shown in Table V.

Isoelectric focusing of cytosolic glutathione S-transferase

Isoelectric focusing (IEF) was used to study the

TABLE V

PHYSICAL AND IMMUNOCHEMICAL PROPERTIES OF GLUTATHIONE *S*-TRANSFERASE ISOENZYMES RESOLVED BY HYDROXYAPATITE HPLC

Property	Peak number		
	1	2 [a]	3
Elution from HPHT (min)	15.5	37.0	51.0
Subunit molecular mass (kDa)	24.8	26.7	26.0
Cross-reactivity with antiserum against:			
ε	−	−	+
μ	−	+	−
λ	+	−	−

[a] Peak 2 protein obtained from liver 7 contained a polypeptide with a molecular mass of 29.0 kDa which did not possess glutathione *S*-transferase activity and did not cross-react with any of the antisera tested.

TABLE IV

PEAKS RESOLVED BY HYDROXYAPATITE HPLC

The amount of protein (A_{280}) present in each peak has been assigned a relative score out of a total of 20.

Peak number	Elution time (min)	Relative size of peak							
		Human liver: 1	2	3	4	5	6	7	8
1	15.5	0	0	0	0	0	0	2	1
2	37.0	11	1	5	8	0	2	3	0
3	51.0	9	19	15	12	20	18	15	19

Fig. 3. The resolution of cytosolic glutathione *S*-transferase using hydroxyapatite HPLC. Enzymes purified by *S*-hexylglutathione affinity chromatography were further resolved by chromatography on Bio-Gel HPHT. The hydroxyapatite column was equilibrated with 10 mM sodium phosphate, pH 6.7, and protein was eluted (0.5 ml/min) with a linear 10–350 mM sodium phosphate gradient. The A_{280} of the column eluate was followed. The proteins eluting at 15.5, 37.0 and 51.0 min are referred to as peak 1, peak 2 and peak 3 respectively. The profiles obtained from material purified from livers 1 (a), 2 (b), 4 (c) and 7 (d) are shown.

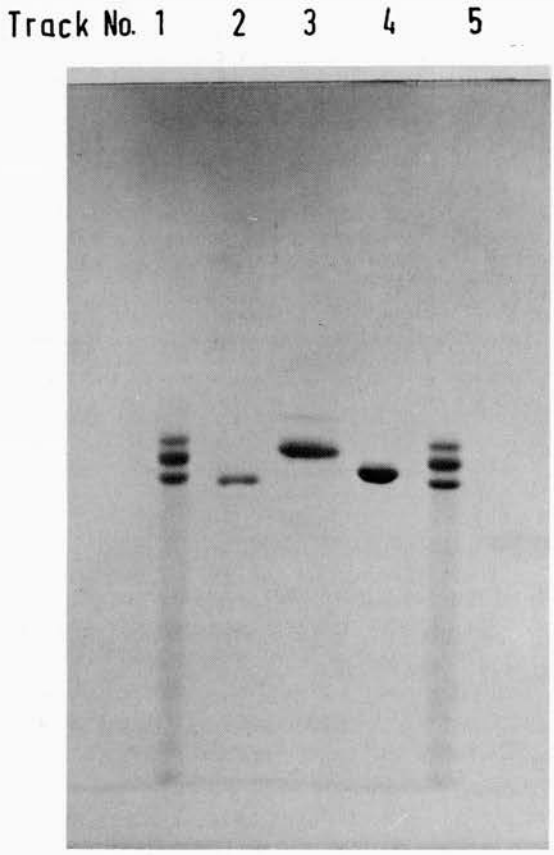

Fig 4. Electrophoretic analysis of glutathione *S*-transferase isoenzymes resolved by hydroxyapatite HPLC. Enzyme subunits resolved by hydroxyapatite were identified by SDS-PAGE. The resolving gel comprised 12.0% (w/v) polyacrylamide and 0.32% (w/v) cross-linker. The gel was loaded as follows: track 1, rat liver affinity-purified glutathione *S*-transferase pool containing Ya (25.5 kDa), Yb (26.3 kDa) and Yc (27.5 kDa) subunit markers; track 2, Bio-Gel HPHT peak 1 from liver 7; track 3, Bio-Gel HPHT peak 2 from liver 3; track 4, Bio-Gel HPHT peak 3 from liver 8; track 5, rat liver glutathione *S*-transferase pool.

polymorphism associated with the isoenzymes; this technique has been employed by several groups of workers to identify the human glutathione *S*-transferase [2,17,19,30]. We have previously used thin-layer IEF in polyacrylamide-gels to show that isoenzymes ε (B_1B_1), δ (B_1B_2), γ (B_2B_2), μ and λ have p*I* values of 8.9, 8.75, 8.4, 6.1 and 4.8, respectively [17]; purified portions of these enzymes were run in parallel with the samples and the marker proteins to facilitate identification of glutathione *S*-transferase in the different livers.

IEF of portions of glutathione *S*-transferase purified, by *S*-hexylglutathione-Sepharose 6B, from the eight liver samples is shown in Fig. 5. Each purified glutathione *S*-transferase pool produced a different electrophoretic pattern. Proteins at the cathodal, or basic, end of the gel were observed in all samples, but were only detected faintly in liver 1. Protein that migrated with glutathione *S*-transferase ε (B_1B_1, p*I* 8.9) was seen in livers 2, 3, 4, 5 and 8, whereas protein that migrated with glutathione *S*-transferase δ (B_1B_2, p*I* 8.75) was detected in all livers but was only faintly observed in livers 1 and 4. Liver 6 is the only liver studied that appeared to contain large amounts of glutathione *S*-transferase γ (B_2B_2, p*I* 8.4). Both livers 5 and 6 contained significant amounts of a further basic protein band (p*I* 8.2); this may correspond to the glutathione *S*-transferase designated transferase β by Kamisaka et al. [30]. In this context it may be noteworthy that livers 5 and 6 were shown by RIA to contain larger amounts of B_2 than other livers.

Although the 'basic' glutathione *S*-transferases were present in all liver samples the 'neutral' and 'acidic' forms were only detected in certain livers. Protein that co-focused with the μ isozyme (p*I* 6.1) was clearly seen in livers 1 and 3 and was also

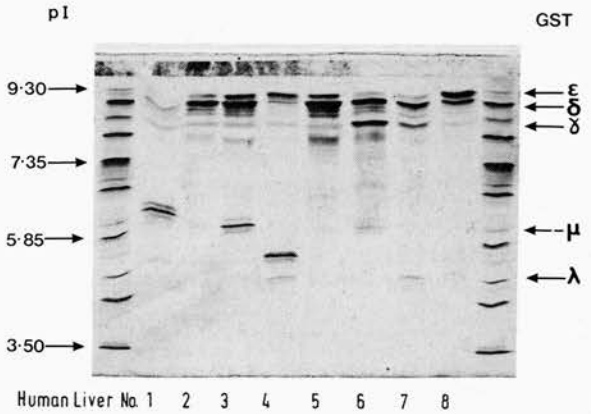

Fig. 5. Isoelectric focusing of cytosolic glutathione *S*-transferase purified by affinity chromatography. IEF was performed using a broad-range gel (pH 3.5–9.5) in thin-layer 5% (w/v) polyacrylamide. The liver samples applied are indicated. The tracks on the extreme left and right contain the p*I* calibration proteins. The purified human glutathione *S*-transferases which were run in parallel focused at the positions shown.

faintly observed in liver 6. Liver 4 was unique as it possessed a strongly stained band of p*I* 5.5 that focused between μ (p*I* 6.1) and λ (p*I* 4.8). Close examination of the patterns from livers 1, 4 7 and 8 revealed very faint bands that co-migrated with λ.

Isoelectric focusing of purified 'neutral' glutathione S-transferase

In order to demonstrate that the novel protein band of p*I* 5.5 in liver 4 represented a glutathione *S*-transferase, a large-scale purification of the transferases in this liver specimen was undertaken; liver 3 was processed in parallel to enable glutathione *S*-transferase μ (p*I* 6.1) to be prepared for comparative purposes. These proteins were purified using, sequentially, DEAE-cellulose, *S*-hexylglutathione-Sepharose 6B, chromatofocusing and hydroxyapatite chromatography as described previously [5,6]. SDS-PAGE showed that the 'neutral' enzymes from livers 3 and 4 were both composed of Yb subunits (26.7 kDa). Both enzymes possessed a similar specific activity for 1-chloro-2,4-dinitrobenzene and both possessed a similar elution position from the Bio-Gel HPHT column. Fig. 6 shows the IEF gel of the 'neutral' glutathione *S*-transferase from liver 3 (p*I* 6.1) and liver 4 (p*I* 5.5) and verifies that the novel protein band in Fig. 5 represents a 'neutral'-type glutathione *S*-transferase.

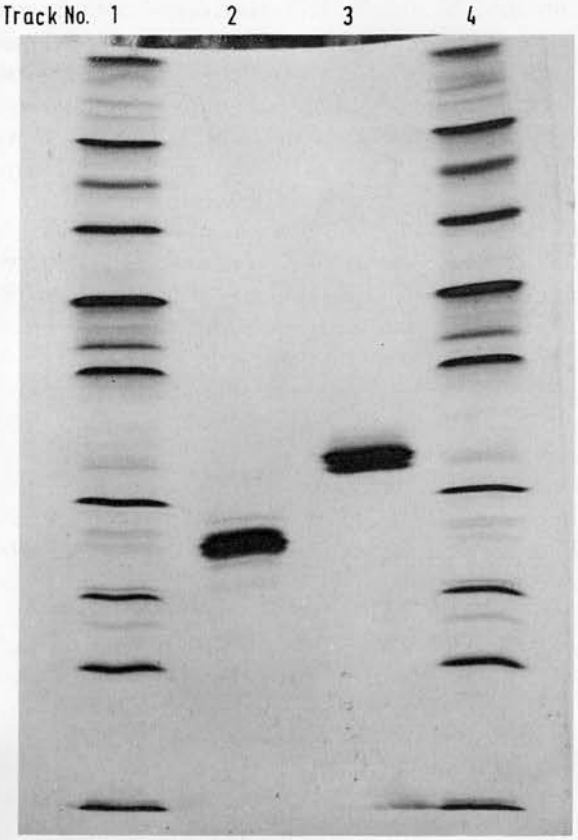

Fig. 6. Isoelectric focusing of purified preparations of 'neutral' glutathione *S*-transferase of p*I* 6.1 and 5.5. IEF was performed using a broad-range gel (pH 3.5–9.5) in thin-layer 5% (w/v) polyacrylamide. The gel was loaded as follows: tracks 1 and 4 contained protein p*I* calibration standards, track 2 'neutral' enzyme from liver 4 and track 3 'neutral' enzyme from liver 3.

Discussion

The cytosolic glutathione *S*-transferases in man have been divided by some researchers into three groups designated 'basic', 'neutral' and 'acidic' [2]; others have referred to the enzymes simply as either 'cationic' or 'anionic' [13,14,31–33]. There is some uncertainty about the structural and immunochemical relationships between the various forms [13,14,17,18,33]; hence it is not clear which of these two classifications best describes the human enzymes.

Our data, like those of Soma et al. [18] and Vander Jagt et al. [34], show that the cytosolic human enzymes can best be divided into three groups. These groups are immunochemically distinct, comprise subunits of different molecular mass and elute at separate positions from hydroxyapatite HPLC columns. Unlike Singh et al. [15], we found no evidence that subunits belonging to glutathione *S*-transferase of different groups (i.e. 'basic' and 'acidic') can hybridize nor did we find that the 'basic' forms each comprise two polypeptides of different molecular mass.

The cytosolic glutathione *S*-transferases have been grouped according to their charge, but the p*I* ranges quoted for the three families are not clear-cut. The fact that the 'basic', 'neutral' and 'acidic' forms comprise Ya subunits (26.0 kDa), Yb subunits (26.7 kDa) and Yf subunits (24.8 kDa), respectively, suggests that these enzymes might be better classified, like the rat glutathione *S*-trans-

ferases, according to their mobility during SDS-PAGE.

Zymogram starch-gel analysis [35] and column isoelectric focusing [19,36] have previously been used to investigate the variations in glutathione *S*-transferase forms expressed in human liver. However, these studies were conducted on crude hepatic cytosols and provided no indication of the structural basis for the differences observed in the glutathione *S*-transferase of different individuals. During the present study the polymorphism associated with human glutathione *S*-transferase has been investigated using electrophoretic methods (SDS-PAGE and IEF), immunochemical techniques (immuno-blotting and RIA), hydroxyapatite HPLC, as well as different substrates to establish whether variations exist in the catalytic profiles of the glutathione *S*-transferase pools. Each of the methods used had advantages and disadvantages.

The approach that provided the most detailed information was the IEF method, performed in thin-layer polyacrylamide gel. However, it was not quantitatively reliable, and required individual purified glutathione *S*-transferase isoenzymes to be available for unequivocal identification of forms. Hydroxyapatite HPLC provided results that were the most quantitatively reliable, and had the advantage of allowing the three families to be prepared separately and analysed independently; however, Bio-Gel HPHT is unable to separate members of the same gene family. Similarly, the immuno-blotting method was unable to discriminate between members of the same family but, in its favour, it was very sensitive and could identify those livers that contained the 'acidic' Yf subunit. RIA for the basic subunits, B_1 and B_2, proved valuable since, unlike immuno-blotting, it could discriminate between the 'basic' Ya subunits and provided information that was in broad agreement with the IEF. To date RIA methods for the 'neutral' or 'acidic' isozymes have not been developed and the technique cannot yet be universally applied to study polymorphism. The catalytic approach to studying polymorphism is limited since, with the exception of 1-chloro-2,4-dinitrobenzene, most substrates lack sensitivity. The use of *S*-hexylglutathione-Sepharose to overcome this problem, by concentrating the glutathione *S*-transferase, was useful when ethacrynic acid was employed to identify the Yf-containing livers. However, this approach may result in the inhibition of activity by *S*-hexylglutathione as appears to be the case when trans-4-phenyl-3-buten-2-one is used to identify the Yb-containing livers.

Board [35] first suggested that the glutathione *S*-transferase forms in man could be ascribed to the existence of several loci, and that the patterns of activity observed were attributable to subunit combinations coded for by three autosomal alleles at locus 1 ('neutral') and two autosomal elleles at locus 2 ('basic'). The view that the variant patterns of the locus 2 enzyme ('basic') on starch-gel are due to allelic variation is not held by all workers [37]. We have previously reported the existence of two basic glutathione *S*-transferase subunits, B_1 and B_2 [17], that might account for the zymogram patterns reported by Board [35] at locus 2. The IEF patterns of the basic glutathione *S*-transferase show that other protein bands exist that are not accounted for by B_1B_1 (p*I* 8.9), B_1B_2 (p*I* 8.75) and B_2B_2 (p*I* 8.4) combinations. It appears likely that further allelic variations exist at locus 2. These extra protein bands have p*I* values 8.2–8.0 and were found in the livers that contained relatively large amounts of the B_2 subunits. It is possible that these additional forms represent B_2B_3 subunit combinations.

Board [35] has reported that the polymorphic glutathione *S*-transferase activity patterns observed for the products of locus 1 ('neutral') can be accounted for by the existence of three alleles, one of which is null. Unfortunately, our present knowledge of the 'neutral' form does not allow the zymogram data to be interpreted at an isoenzyme level. Warholm et al. [12] characterised a 'neutral' enzyme, glutathione *S*-transferase μ, and showed that it is only expressed in about 60% of individuals. Since these workers have not reported any heterogeneity in preparations of the μ isozyme, it is difficult to relate this body of work to that of Board [35]. Our IEF data show that a variant form with p*I* 5.5 existed in liver 4 that was distinct from μ (p*I* 6.1) and λ (p*I* 4.8). This new isoenzyme was isolated by hydroxyapatite HPLC and shown to be composed of subunits of the same size as those of glutathione *S*-transferase μ (26.7 kDa). These subunits also cross-reacted with antisera against

the μ form when analysed by immuno-blotting (Fig. 2b). A large-scale preparation of this glutathione *S*-transferase of p*I* 5.5 has been undertaken and the enzyme was found to possess similar, though not identical, activities to glutathione *S*-transferase μ. It appears highly probable that this 'new' form represents an allelic variant at locus 1, but its subunit composition and extent of sequence homology with glutathione *S*-transferase μ await further study.

In the series of livers examined none contained large concentrations of the 'acidic' enzyme. It was present in measurable amounts in several samples (livers 7 and 8) but was absent from the majority of livers investigated.

The present study has demonstrated that the human glutathione *S*-transferases are subject to extensive polymorphism. The variations in the content of different livers are such that it is desirable to perform an initial 'screening' of livers before undertaking a glutathione *S*-transferase purification. In our opinion a small-scale purification on *S*-hexylglutathione-Sepharose 6B, followed by both IEF and immunoblotting, should be sufficient to establish which livers should be processed to allow the isolation of specific isoenzymes. This approach should save considerable time during the work-up of suitable livers to allow the study of the protein chemistry of the enzymes encoded by the glutathione *S*-transferase loci 1, 2 and 3; these correspond to the neutral (Yb-containing), the basic (Ya-containing) and the acidic (Yf-containing) forms, respectively. Glutathione *S*-transferase nomenclatures are listed in Table VI, along with an alternative designation of the different classes [38] which allows for the fact that the p*I* values of the different classes are not conserved between species.

TABLE VI

LITERATURE NOMENCLATURES OF HUMAN GLUTATHIONE *S*-TRANSFERASES (GST)

Class of human enzyme [a]	Zymogram [b] analysis (loci designation)	Isoenzyme [c]	Subunit type [d]	Quaternary structure [c]
Basic (α)	GST 2	γ	Ya	B_2B_2
		δ	Ya	B_1B_2
		ε	Ya	B_1B_1
Neutral (μ)	GST 1	μ	Yb	
Acidic (π)	GST 3	λ	Yf	

[a] The α, μ and π classes were proposed by Mannervik et al. [38] as an alternative designation to define the different multi-gene families.

[b] Board [35].

[c] Stockman et al. [17].

[d] Subunit types defined by cross-reactivity with antisera raised against rat glutathione *S*-transferase L (YaYa), A (Yb_1Yb_1) and H (YfYf) [25,39].

Acknowledgements

We gratefully acknowledge the generous help of Dr. C.R. Wolf and his colleagues in the ICRF laboratory, Department of Biochemistry, University of Edinburgh, in providing samples of fresh liver and the data on the patients' histories. This work was supported by grants from the Scottish Home and Health Department (K/MRS/50/C570) and the Medical Research Council (G8126392 SB). We thank Professor L.G. Whitby for critically reading this script and Mrs. E. Ward for her secretarial assistance.

References

1 Jakoby, W.B. (1978) Adv. Enzymol. Relat. Areas Mol. Biol. 46, 383–414

2 Mannervik, B. (1985) Adv. Enzymol. Relat. Areas Mol. Biol. 57, 357–417

3 Chasseaud ,L.F. (1979) Adv. Cancer Res. 29, 175–274

4 Smith, G.J., Ohl, V.S. and Litwack, G. (1977) Cancer Res. 37, 8–14

5 Hayes, J.D. (1984) Biochem. J. 224, 839–852

6 Hayes, J.D. (1986) Biochem. J. 233, 789–798

7 Hayes, J.D., Strange, R.C. and Percy-Robb, I.W. (1981) Biochem. J. 197, 491–502

8 Beale, D., Ketterer, B., Carne, T., Meyer, D.J. and Taylor, J.B. (1982) Eur. J. Biochem. 126, 459–463

9 Boyer, T.D., Kenney, W.C. and Zakim, D. (1983) Biochem. Pharmacol. 32, 1843–1850

10 Awasthi, Y.C. and Singh, S.V. (1985) Comp. Biochem. Physiol. 82B, 17–23

11 Hayes, J.D. and Mantle, T.J. (1986) Biochem. J. 233, 779–788

12 Warholm, M., Guthenberg, C. and Mannervik, B. (1983) Biochemistry 22, 3610–3617

13 Koskelo, K. and Icen, A. (1984) Scand. J. Clin. Lab. Invest. 44, 159–162

14 Awasthi, Y.C., Dao, D.D. and Saneto, R.P. (1980) Biochem. J. 191, 1–10

15 Singh, S.V., Dao, D.D., Partridge, C.A., Theodore, C., Srivastava, S.K. and Awasthi, Y.C. (1985) Biochem. J. 232, 781–790
16 Dao, D.D., Partridge, C.A., Kurosky, A. and Awasthi, Y.C. (1984) Biochem. J. 221, 33–41
17 Stockman, P.K., Beckett, G.J. and Hayes, J.D. (1985) Biochem. J. 227, 457–465
18 Soma, Y., Satoh, K. and Sato, K. (1986) Biochim. Biophys. Acta 869, 247–258
19 Warholm, M., Guthenberg, C., Mannervik, B., von Bahr, C. and Glaumann, H. (1980) Acta Chem. Scand. B34, 607–610
20 Warholm, M., Guthenberg, C., Mannervik, B. and von Bahr, C. (1981) Biochem. Biophys. Res. Commun. 98, 512–519
21 Mannervik, B. and Guthenberg, C. (1981) Methods Enzymol. 77, 231–235
22 Habig, W.H. and Jakoby, W.B. (1981) Methods Enzymol. 77, 398–405
23 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
24 Laemmli, U.K. (1970) Nature (London) 227, 680–685
25 Hayes, J.D. and Mantle, T.J. (1986) Biochem. J. 237, 731–740
26 Towbin, H., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354
27 Hayes, J.D., Gilligan, D., Chapman, B.J. and Beckett, G.J. (1983) Clin. Chim. Acta 134, 107–121
28 Beckett, G.J. and Hayes, JD. (1984) Clin. Chim. Acta 141, 267–273
29 Hayes, J.D., Coulthwaite, R.E., Stockman, P.K., Hussey, A.J., Mantle, T.J. and Wolf, C.R. (1986) Arch. Toxicol., in the press
30 Kamisaka, K., Habig, W.H., Ketley, J.N., Arias, I.M. and Jakoby, W.B. (1975) Eur. J. Biochem. 60, 153–161
31 Pattinson, N.R. (1981) Biochem. Biophys. Res. Commun. 102, 403–410
32 Koskelo, K. and Valmet, E. (1980) Scand. J. Clin. lab. Invest. 40, 179–184
33 Koskelo, K. (1983) Scand. J. Clin. Lab. Invest. 43, 133–139
34 Vander Jagt, D.L., Hunsaker, L.A., Garcia, K.B. and Royer, R.E. (1985) J. Biol. Chem. 260, 11603–11610
35 Board, P.G. (1981) Am. J. Hum. Genet. 33, 36–43
36 Sherman, M., Titmuss, S. and Kirsch, R.E. (1983) Biochem. Int. 6, 109–118
37 Strange, R.C., Faulder, C.G., Davis, B.A., Hume, R., Brown, J.A.H., Cotton, W. and Hopkinson, D.A. (1984) Ann. Hum. Genet. 48, 11–20
38 Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M.K., Warholm, M. and Jornvall, H. (1985) Proc. Natl. Acad. Sci. USA 82, 7202–7206
39 Hayes, J.D., McLellan, L.I., Stockman, P.K., Chalmers, J., Howie, A.F., Hussey, A.J. and Beckett, G.J. (1987) in 10th European Drug Metabolism Workshop, Francis & Taylor, in the press